THE METABOLIC ROLE OF REGENERATING ISLET-DERIVED 2 AND DIETARY SELENIUM IN MICE OVEREXPRESSING CELLULAR GLUTATHIONE PEROXIDASE 1

A Dissertation

Presented to the Faculty of the Graduate School of

Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

By

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May 2014

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Cornell University 2014

Previously, we found that mice overexpressing glutathione peroxidase 1 (GPX1) (OE) developed type 2 diabetes mellitus (T2DM)-like phenotypes and hyperinsulinemia and hyper-secretion of insulin after glucose stimulation were two primary effects of the cellular GPX1 overproduction. To understand how GPX1 overexpression led to these primary metabolic outcomes, we identified an islet protein, regenerating islet-derived 2 (Reg2), whose mRNA and protein level in islets were diminished by GPX1 overproduction. We hypothesized that the depleted REG2 in OE islets mediated the OE T2DM-like phenotypes. Firstly, we demonstrated that the biochemical regulation of REG2 by GPX1 was through modulating intracellular redox status both *in vitro* and *in vivo*. We then demonstrated a novel metabolic function of REG2 in rescuing several phenotypes in the OE mice including hyperinsulinemia, hyper-secretion of insulin after the glucose stimulation, hyperglycemia and hypertriglyceridemia. We further found that REG2 suppressed insulin secretion in a glucose-dependent way, and that glucose metabolism served as the downstream signaling *in vitro*. In addition, we uncovered a potential functional domain in REG2, the C-type lectin domain, which might participate in the inhibition of glucose-stimulated insulin secretion (GSIS) by REG2.

Recent human and animal studies have shown that selenium (Se) supplements lead to increased risk of developing T2DM. However, the underlying mechanisms are not well understood. Since GPX1 is the most abundant selenoprotein in the body, the OE mice might serve as a good animal model to study the effects of Se supplements. To determine if dietary Se deficiency could improve the T2DM-like phenotypes in OE mice, we fed WT and OE mice a Se-deficient diet or Se-supplemented diet for 4 months. We showed that dietary Se deficiency in the OE mice partially alleviated the T2DM-like phenotypes. Expression of genes and proteins related to insulin synthesis and secretion, as well as glycolysis, gluconeogensis and lipogenesis was down-regulated by dietary Se deficiency.

Taken together, these new insights into the regulation and function of Reg2 and Se have not only led to their identifications as important players in the diabetogenic risk of GPX1 overexpression and(or) Se supplements, but also raise the possibility to developing strategies to combat T2DM.

BIOGRAPHICAL SKETCH

Xi Yan was born and raised in Chongqing, China, a beautiful city that is famous for spicy food. Her parents are Mr. Fangkai Yan and Dr. Xiao Zhou. Under the influence of her mom, who is a physician, Xi has shown great interest in medicine and science since childhood. After high school, she joined the clinical medicine program in the Medical College of Jinan University in Guangzhou, China, and received her Bachelor of Medicine degree (equivalent to M.D. in the U.S.A.) in 2007. During her undergraduate years in Guangzhou, Xi enjoyed the intense medical training, as well as diverse student activities. She was also exposed to scientific research for the first time when she volunteered to work as an undergraduate research assistant there. With tremendous curiosity and enthusiasms of scientific discovery, Xi joined Professor Xingen Lei's lab and is pursuing the Doctor of Philosophy degree in the Department of Animal Science at Cornell University. In the Lei lab, Xi focused on the study of the metabolic function of dietary Se supplement and REG2 protein in GPX1-overexpressing mice using mice, primary cells and mammalian cell lines as models. She leveraged protein engineering, molecular biology and biochemical techniques, as well as cell culture and mouse handling approaches, to study the novel functions and molecular mechanisms of Se and REG2 protein. Love of spicy food seems to have helped her build outgoing personality and brought her lots of life-long friendship from the Cornell community. Xi feels extremely fortunate to meet her beloved husband, Ryan Lau, who is in the Ph.D. program of Electrical and Computer Engineering department in Cornell University. After four-year marathon, they got engaged in Cornell plantation and married on the top of Cornell McGraw Tower. Xi completed her Doctor of Philosophy in Animal Science with minors in Pharmacology, and Molecular and Integrative Physiology in February of 2014.

To my family, mentors and friends

ACKNOWLEDGEMENTS

I have been very fortunate to have tremendous help and support from the outstanding and kind people around me, to only some of whom it is possible to give particular mention here.

Foremost, I would like to express my sincerest gratitude to my Ph.D. advisor, Prof. Xin Gen Lei, for his excellent guidance, caring, patience and support in the past six years. Sincerely thanks for taking me as your student and providing me such a great opportunity to work on the challenging and exciting research projects. Without your tremendous encouragement and guidance, I would never have become a mature scientist and an independent thinker as I am today.

Besides my advisor, I would like to show my endless appreciation to the rest of my thesis committees: Prof. Ling Qi, Prof. Robin Davisson and Prof. Charles McCormick, who are all brilliant scientists in their own fields. I am truly honored to have had them on my committees. I am very grateful for their knowledgeable advice, insightful comments, encouragement, and recommendation during my study at Cornell. Prof. McCormick is one of the kindest and knowledgeable persons that I have ever met. I really appreciate his encouragement and help on my research. Prof. Qi has been invaluable on both an academic and a personal level, for which I am extremely grateful. I thank Prof. Davisson for challenging me and nurturing my growth as a mature scientist.

I also feel very lucky to get lots of help from the Lei lab. I thank all the present and past members. I will forever be thankful to Dr. Marko Z. Vatamaniuk, Carol A. Roneker and Dr. Jeremy Weaver for their kind help and support all these years. Their enthusiasm and love for

science are contagious. I have learned a lot from them, not only science but also how to become a kind and responsible person. I am also very grateful to Zeping Zhao, Xinhui Wang, Dr. Jun Won Yun, Dr. Ricardo Ekmay, Krystal K. Lum, Dr. Jonggun Kim, Dr. Yixuan Zhu, Matthew Barcus, Dr. Stephanie Gatrell and Joanne G. Parsons, for their friendship and helpful advice.

I extend my gratitude to Prof. Qiaoming Long, Drs Li Li, Fenghua Hu, Xiaolai Zhou and Xiangjun Zhou for their generous technical assistances and guidance through our collaboration. I am also very grateful to Prof. Pat Johnson for all her kind help. Thanks to all my dear friends, especially Iris Sun, Weishan Huang, Jingwen Zhang, Victor Tse and Jean K. Millet.

Above all, I would like to specially thank my husband, Ryan Lau, for his endless love, great patience and full support at all times. I also want to thank my parents, my parents in law and my brother in law, Roger Lau, who have given me their unequivocal love and support throughout, as always.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACC1	Acetyl- Coenzyme A Carboxylase
AKT	Rac-Alpha Serine/Threonine-Protein Kinase
AP-1	Activator Protein-1
BETA2	Neurogenic Differentiation 1
CARS	Cysteinyl-Trna Synthetase
CAT	Catalase
CDC	Centers for Disease Control and Prevention
CFOS	Fbj Murine Osteosarcoma Viral Oncogene Homolog
CLECT	Reg2 C-Type Lectin Domain
CTLD	C-Type Lectin Domain
CTRL	Control
CTRL	Reg2 C-Terminal
CYP7A1	Cholesterol 7 Alpha-Hydroxylase
DM	Diabetes Mellitus
DQ	Diquat
EGFR	Epidermal Growth Factor Receptor
EXTL3	Exostoses Tumor-Like Gene 3
F1,6BP	Fructose 1,6-Bisphosphate
FASN	Fatty Acid Synthase
FOXA2	Forkhead Box A2
FOXO1	Forkhead Box O1
G6P	Glucose-6-Phosphate
GK	Glucokinase

GLUT2	Glucose Transporter 2
GPX1	Glutathione Peroxidase 1
GSH	Glutathione
GSIS	Glucose Stimulated Insulin Secretion
GSN	Gelsolin
GTT	Glucose Tolerance Test
H_2O_2	Hydrogen Peroxide
HMGCS2	3-Hydroxy-3-Methylglutaryl-Coenzyme A Synthase 2
HNF1A	Hnf1 Homeobox A
HNF4A	Hepatic Nuclear Factor 4, Alpha
HNRNPU	Heterogeneous Nuclear Ribonucleoprotein U
HPRT	Hypoxanthine-Guanuine Phosphoribosyltransferase
I.P.	Intraperitoneal
IFNγ	Interferon-Gamma
IGF1	Insulin-Like Growth Factor 1
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-22	Interleukin-22
INS1	Insulin 1
IP	Immunoprecipitation
IRS1	Insulin Receptor Substrate 1
IRS2	Insulin Receptor Substrate 2
ITGα5	Integrin Alpha 5
ITGβ1	Integrin Beta 1
ITT	Insulin Tolerance Test

JUND	Jun Proto-Oncogene Related Gene D
KIR6.2	Potassium Inwardly Rectifyling Channel, Subfamily J, Member 11
MAFA	V-Maf Musculoaponeurotic Fibrosarcoma Oncogene Family, Protein A
MCCC1	Methyl Crotonyl Coenzyme A Carboxylase 1
MODY	Maturity Onset Diabetes of The Young
NAC	N-Acetyl Cysteine
NCKAP1	Nck-Associated Protein 1
NEFA	Nonesterified Fatty Acid
NTRL	Reg2 N-Terminal
OE	Glutathione-Peroxidase-1 Overexpressing
P53	Transformation Related Protein 53
PDX1	Pancreatic And Duodenal Homeobox Factor 1
PEPCK	Phosphoenolpyruvate Carboxykinase
PI3K	Phosphatidylinositol 3-Kinase, Regulatory Subunit, Polypeptide 1 Isoform 2
PKC	Protein Kinase C
PPARγ	Peroxisome Proliferator Activated Receptor Gamma
PREGLUC	Preproglucagon
Q-PCR	Real-Time Pcr
RDA	Recommended Dietary Allowance
REG2	Regenerating Islet-Derived Protein 2
ROS	Reactive Oxygen Species
Se	Selenium
Sec	Selenocysteine
SEC23B	Sec23 Homolog B
SECIS	Sec Insertion Sequence

SIRT1	Sirtuin 1
SOD	Superoxide Dismutase
SREBP1a	Sterol Regulatory Element Binding Transcription Factor 1a
SREBP2	Sterol Regulatory Element Binding Factor 2
STAT	Signal Transducer and Activator Of Transcription
STZ	Streptozotocin
SUR1	Sulfonylurea Receptor1
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TG	Triglyceride
TNFα	Tumor Necrosis Factor-Alpha
UCP2	Uncoupling Protein 2
VCP	Valosin-Containing Protein
WT	Wild-Type

CHAPTER 1

Overview

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a chronic endocrine disorder characterized by a persistent elevation of plasma glucose levels. According to the National Diabetes Fact Sheet from the Centers for Disease Control and Prevention (CDC) in 2011, nearly 26 million Americans have diabetes, and another 79 million U.S. adults have prediabetes. Diabetes is a public health burden as approximately $175 billion was annually spent on the treatment of the diseases.

DM is classified into the following classes: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes, monogenic forms of diabetes, and other rare forms of this disease (Mellitus, 2003). T1DM is characterized by insulin deficiency due to the loss of pancreatic β cells. It is an autoimmune disease in which the inflammatory reaction attacks and damages β cells, thus causing them to produce insufficient insulin (O'Rahilly, 2009). T2DM is characterized by insulin resistance, a condition in which body tissues do not respond to insulin (Stumvoll and Häring, 2001). It is often accompanied by insulin secretory defect, impaired glucose tolerance, hyperinsulinemia, and dyslipidemia (O'Rahilly, 2009; Stumvoll and Häring, 2001). Gestational diabetes is characterized glucose intolerance during pregnancy, which is usually temporary and reversible (Mellitus, 2003). Other specific types of diabetes include maturity onset diabetes of the young (MODY) (Tattersall, 1998), congenital diabetes, cystic fibrosis, and steroid-related diabetes (Mellitus, 2003).

The most widely spread form of diabetes is T2DM (Rösen et al., 2001). In healthy individuals, pancreatic β cells secrete insulin that travels through the bloodstream to enhance glucose uptake in skeletal muscles, inhibit glucose production in the liver, and inhibit lypolysis in adipose tissue (Fig. 1.1). For individuals with T2DM, the cells in the insulin-sensitive peripheral tissues are resistant to insulin, resulting in glucose intolerance and an increased fasting glucose level as the glucose is unable to enter the body effectively (Fig. 1.1). During the early stages of T2DM, the amount of insulin released by pancreatic β cells increases fourfold to fivefold to adapt to insulin resistance and allow more glucose to enter the body (Kahn et al., 2006). This results in hyperinsulinemia that is characterized by abnormally elevated insulin circulating in the bloodstream. During the late stages of T2DM, persistent hyperinsulinemia eventually exhausts the β cells and causes a continued decline in β cell functionality, which results in insulin deficiency and further contributes to the pathogenesis of T2DM (Lei and Vatamaniuk, 2011).

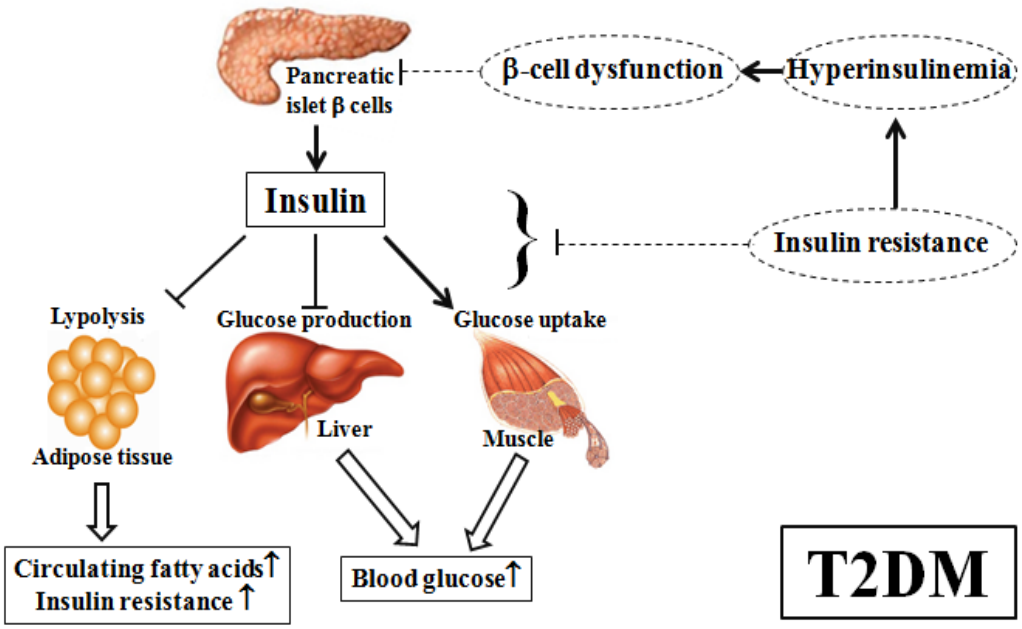


FIGURE 1.1 Type 2 diabetes mellitus. The diagram is based on http://infoaboutdiabetes.com/diabetes-information/

1.2 Hyperinsulinemia

Hyperinsulinemia is seen in an increasing number of people (Hopper et al., 2012). Hyperinsulinemia is strongly associated with metabolic diseases, such as T2DM (Arslanian et al., 2002; Haffner and Miettinen, 1997; Haffner et al., 1992; Koopmans et al., 1997), non-alcoholic fatty liver disease (Hurjui et al., 2012; Kawano and Cohen, 2013; Pirgon et al., 2013; Titov et al., 2012), obesity and hypertension (Modan et al., 1985). Primary hyperinsulinemia turned out to be the strongest predictor of dysglycemia development in a 24-year follow-up human study (Dankner et al., 2009). Transgenic hyperinsulinemic mice with 8 or 32 extra copies of the human insulin gene exhibited increased insulin resistance and hypertriglyceridemia, with significantly elevated plasma insulin and reduced insulin receptor binding (Marban et al., 1989; Roth et al., 2004). Alleviation of hyperinsulinemia can prevent or improve the development of metabolic diseases. Lowering insulin through pancreatic-specific knockout of the *insulin 1 (Ins1)* gene prevented high-fat diet-induced fat growth and obesity without causing insulin resistance in adult mice (Mehran et al., 2012). Blocking insulin secretion with diazoxide promoted weight loss in obese humans and rats with increased basal metabolic rate and fat oxidation, but not hyperglycemia (Alemzadeh et al., 2008; Alemzadeh et al., 1998).

1.3 Insulin Synthesis and Secretion

The islets of Langerhans constitute 1 to 2% of the mass of the pancreas and are endocrine cell conglomerates that contain hormone-producing cells such as insulin-secreting β cells. A majority of diabetes research focuses on the differentiation, function, and maintenance of pancreatic β cell mass (Sarsour et al., 2009). When insulin synthesis, secretion or β cell mass is affected, blood insulin level will be consequently altered and eventually result in the development of diabetes.

However, β cell function rather than β cell mass seems to be more crucial in the etiology of T2DM (Ashcroft and Rorsman, 2012). 50% pancreatectomy had only small effect on glucose tolerance, implying around 40% of β cells would be sufficient to maintain glycemia (Menge et al., 2008). Increased insulin secretion and improvement of diabetes by glucagon-like peptide-1 (GLP-1) demonstrated the importance of β cell function in established T2DM (Ashcroft and Rorsman, 2012). β cell dysfunction is one of the events in the progression of T2DM (Sarsour et al., 2009). The reason why β cells are very susceptible to oxidative stress-induced damage is related to a very low intrinsic antioxidant capability (Grankvist et al., 1981).

β cell function is reflected by its capacity to synthesize and secrete insulin. Maintaining normal insulin transcription is important for insulin production. Many transcription factors can bind to insulin gene promoter regions and regulate an insulin biosynthesis, such as pancreatic duodenal homeobox 1 (PDX1), neurogenic differentiation 1 (BETA2), and forkhead box A2 (FOXA2) (Melloul et al., 2002). PDX1 plays a pivotal role in pancreatic β cell function (Ahlgren et al., 1998). BETA2 is crucial for islet β cell maturation, maintenance, and insulin biosynthesis (Anderson et al., 2009b). FOXA2 plays a central role in β cell development by binding to and transactivating Pdx1 (Gao et al., 2008).

Besides insulin synthesis, insulin secretion is another key component in determining insulin levels in the bloodstream. Glucose is an important secretagogue of insulin secretion. When the level of blood glucose rises, glucose is rapidly transported into the cell by glucose transporter 2 (GLUT2), which plays an important role in glucose signaling, insulin secretion, and biosynthesis (Guillam et al., 2000; Valera et al., 1994) (Fig. 1.2). The intracellular glucose is then

phosphorylated to glucose-6-phosphate (G6P) by glucokinase (GK) (German, 1993) (Fig. 1.2). Glucose is metabolized in glycolysis and then in a form of acetyl-CoA enters the TCA cycle (Fig. 1.2). The followed mitochondrial events result in increased respiration, oxidative phosphorylation and ATP synthesis, an increased ATP/ADP ratio, a closure of ATP-sensitive K^+ channels, an opening of the voltage-sensitive Ca^{2+} channels and an influx of extracellular Ca^{2+}, which results in insulin exocytosis (Fig. 1.2). During ATP synthesis in the mitochondria, a proton circuit across the mitochondrial inner membrane drives oxidative phosphorylation, coupling substrate oxidation and ADP phosphorylation (Lowell and Shulman, 2005). This process is positively associated with mitochondrial membrane potential (Mitchell, 1961). Uncoupling protein 2 (UCP2) is a mitochondrial anion carrier protein that increases proton leak across the mitochondria inner membrane. UCP2 dissipates energy stored in the form of the electrochemical potential gradient away from ATP synthase, decreases mitochondrial inner membrane potential and ATP/ADP ratio, and thus attenuates insulin release from pancreatic β cells (Dalgaard, 2012; Lowell and Shulman, 2005; Zhang et al., 2001).

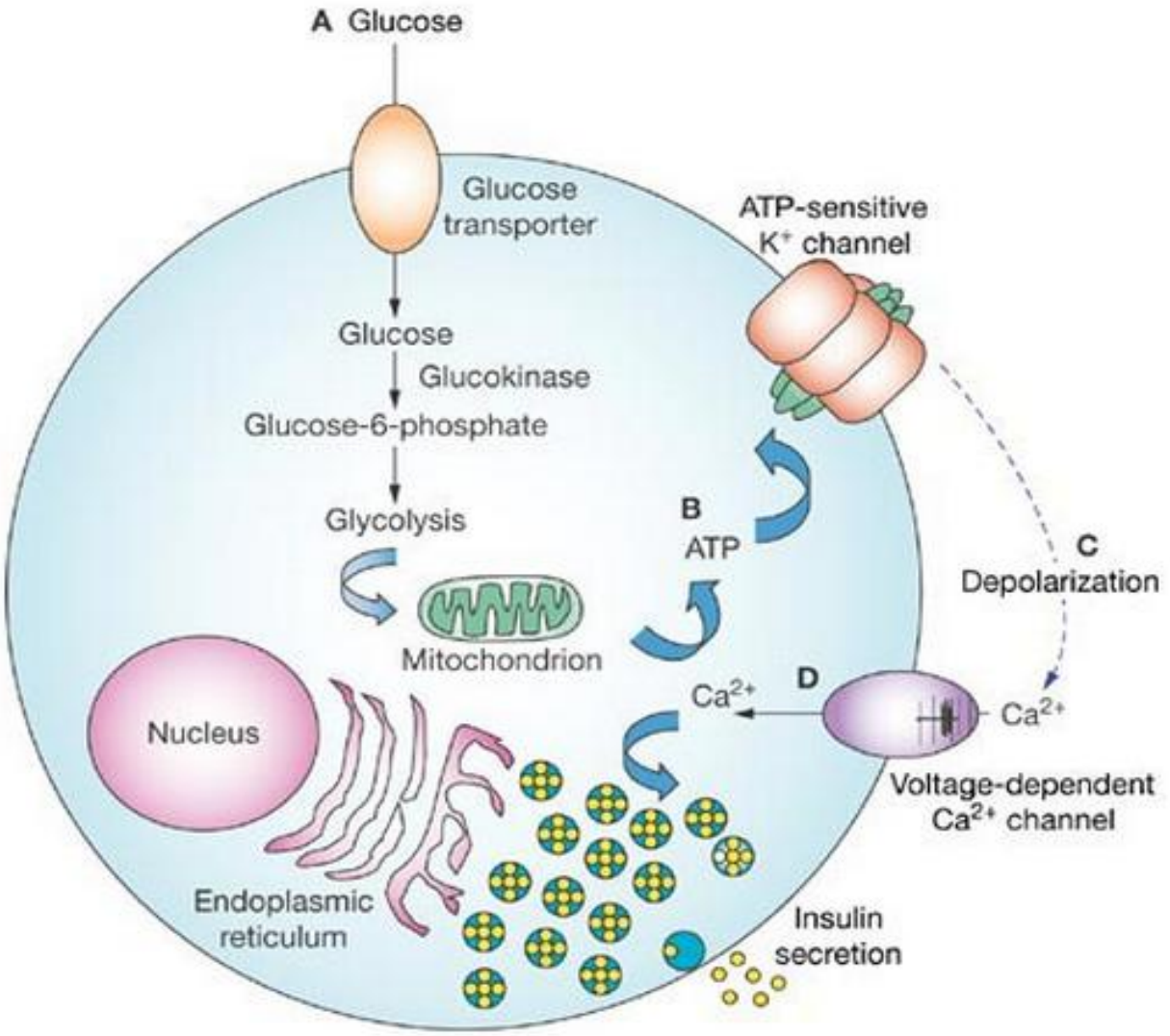


FIGURE 1.2 Glucose-stimulated insulin secretion in a pancreatic β cell. Adopted from (De León and Stanley, 2007).

1.4 Reactive Oxygen Species (ROS) and Antioxidant Defenses

ROS are toxic oxygen-containing molecules that are mainly produced by the mitochondrial electron-transport chain and oxygen-metabolizing enzymatic reactions (Sarsour et al., 2009). ROS consist of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$), and they are highly reactive in redox reactions. An excessive intracellular generation of ROS results in oxidative stress and significant impairment of cell structures, as ROS can induce damage of DNA, proteins, and lipids (Buechter, 1988). It is also perceived that ROS contribute to various human diseases including T2DM, metabolic syndrome, cancer, cardiovascular diseases, neurodegeneration, as well as aging (Chung et al., 2013; Kim et al., 2008; Sarsour et al., 2009).

In T2DM, glucotoxicity from persistent hyperglycemia causes the overproduction of ROS (Poitout and Robertson, 2008). An elevated intracellular ROS can easily impair insulin synthesis or secretion in the β cells, resulting in the deterioration of glucose metabolism and eventually leading to diabetes (Evans et al., 2003). Chronic overconsumption of a high-sucrose or high-fat diet significantly alters ROS production and is highly associated with insulin resistance and diabetes in mice and humans (Anderson et al., 2009a; Bonnard et al., 2008; Mohanty et al., 2002). H_2O_2 can inhibit insulin signaling by blocking insulin-induced tyrosine phosphorylation of insulin receptor β subunit in fibroblast cell lines and adipocytes (Hansen et al., 1999), and by decreasing insulin-induced RAC-alpha serine/threonine-protein kinase (AKT) phosphorylation in vascular smooth muscle cells (Gardner et al., 2003). Reduction of the intracellular ROS level significantly improves insulin sensitivity in mice fed a high-fat diet (Anderson et al., 2009a).

Paradoxically, it has become apparent that H_2O_2 can also function as signaling molecule to facilitate insulin signaling and improve metabolic syndrome. Czech et al. suggested that H_2O_2 serves as an insulin mimetic in adipocytes (Czech et al., 1974), while May and colleagues further demonstrated its role in the transduction of insulin signals based on increased pyruvate dehydrogenase activity and lipid synthesis from glucose in adipocytes (May and de Haën, 1979). Mahadev et al. found that H_2O_2 enhanced insulin signaling through the PI3K/AKT pathway in adipocytes (Mahadev et al., 2001), and most recently, Loh and co-workers showed that ROS increased insulin sensitivity through ROS/PI3K/AKT signaling in muscle under both *in vivo* and *in vitro* conditions (Loh et al., 2009).

In order to detoxify ROS and maintain cellular redox homeostasis, mammals have three cellular antioxidant defense systems: 1) antioxidant enzymes, such as GPX1, superoxide dismutase (SOD), and catalase (CAT); 2) non-enzymatic compounds like vitamin E, vitamin C, uric acid, and glutathione (GSH); 3) sequestration and repairing systems (Lei and Vatamaniuk, 2011). Pancreatic β cells are considered to be low in free radical quenching (antioxidant) enzymes and thus more susceptible to oxidative stress (Grankvist et al., 1981). The health benefits of antioxidants are well known, and more than 30% of the U.S. population takes antioxidants from daily supplements of vitamins and minerals (Loya et al., 2009; Millen et al., 2004). However, a number of recent animal studies and clinical trials have failed to prove that antioxidants played a beneficial role in preventing or treating T2DM (Chen et al., 2005; Czernichow et al., 2006; Li et al., 2006; Liu et al., 2006; McClung et al., 2004; Song et al., 2009). Global overexpression of an antioxidant enzyme, GPX1, led to the development of insulin resistance, hyperglycemia, hyperinsulinemia and obesity in mice (McClung et al., 2004).

1.5 Se, Selenoproteins, and GPX1

Se is an essential trace element that serves as an important antioxidant in human and animal nutrition. The recommended dietary allowance (RDA) for Se is 55 μg/day for adults. Se deficiency is associated with Keshan disease (Chen et al., 1980), Kashin-Beck disease (Xu et al., 1991), and Behcet's disease (Esalatmanesh et al., 2011). The role of Se in preventing cancer has been of great interest, as Se supplements have reduced the incidence of several types of carcinoma (Clark et al., 1996). In addition, Se is considered to be anti-diabetic (Faure, 2003; Mueller and Pallauf, 2006) due to its insulin-mimetic effect (Ezaki, 1990). However, a number of recent human studies have shown a hyperglycemic, hyperlipidemic, and pro-diabetic effect from Se supplements (Bleys et al., 2007; Bleys et al., 2008; Laclaustra et al., 2010; Stranges et al., 2010a; Stranges et al., 2007; Stranges et al., 2010b). In addition, the Se and Vitamin E Cancer Prevention Trial (SELECT) study found a correlation between Se supplements and the increased development of T2DM in men (Lippman et al., 2009). In line with these studies, we found that a prolonged feeding of high dietary Se (3 mg/kg) induced mild gestational diabetes in the first-parity pregnant rats and insulin resistance in their offspring (Zeng et al., 2012). Meanwhile, Labunskyy and collegues have also shown similar effects of Se on body glucose and lipid metabolism (Labunskyy et al., 2011).

The functional moiety of Se in selenoproteins is selenocysteine (Sec), as the antioxidant properties of Se appear through the co-translational insertion of Sec in selenoproteins. This insertion requires a UGA termination codon (Chambers et al., 1986) and Sec insertion sequence (SECIS) elements (Berry et al., 1991) to complete translation. Sec is identical to cysteine except that it contains a Se atom instead of sulfur. The Se atom is typically ionized at physiological pH

(Jacob et al., 2003) and is critical for proper selenoprotein functionality (Gasdaska et al., 1999; Lee et al., 2000). The SECIS element is a stem-loop structure that resides downstream of the UGA codon in the 3'-untranslated regions of eukaryotic selenoprotein transcripts. The recognition and insertion of the UGA stop codon as a Sec codon requires the presence of SECIS, $tRNA^{Sec}$, a Sec-specific elongation factor, and SECIS-binding proteins (Caban and Copeland, 2006).

To date, twenty-five selenoproteins are identified in humans. Their biochemical activities are involved in oxidoreduction, selenocysteine synthesis and Se transport (Moghadaszadeh and Beggs, 2006). Six forms of glutathione peroxidase (GPX) have been identified so far, and four of them (GPX1, GPX2, GPX3 and GPX4) contain Se in the form of selenocysteine residue (Forstrom et al., 1978). Among the four GPXs containing selenocysteine, GPX1 is the most abundant form, which incorporates four atoms of Se per 84 kDa (Rotruck et al., 1973). GPX1 is considered to be the main storage form of body Se (Burk, 1991), which may be the reason why its mRNA expression and enzyme activity are more susceptible to dietary Se deficiency than other selenoproteins (Bermano et al., 1995).

GPX1 is the first identified selenoprotein and is an antioxidant enzyme that catalyzes the reduction of H_2O_2 to water (H_2O) using GSH as a reducing agent in erythrocytes (Mills, 1957) and other types of cells. Using the GPX1 knockout mouse model, our lab had previously demonstrated the protection role of GPX1 on paraquat-induced depletion of NADPH and NADH (Cheng et al., 1999a), diquat-induced lethal oxidative stress (Fu et al., 1999), as well as ROS-mediated oxidative injuries (Fu et al., 2001). Furthermore, this role of GPX1 cannot be replaced

by a high level of dietary vitamin E supplement (Cheng et al., 1999b). These studies are in line with other research that has demonstrated the protective role of GPX1 in myocardial and brain ischemia reperfusion injury (Crack et al., 2001; Yoshida et al., 1997).

1.6 Regenerating Islet-Derived Protein (Reg) Family Proteins

The Reg family proteins have been studied for over 25 years (Terazono et al., 1988). There are seven identified Reg proteins in mice, five in humans, and five in rats (Parikh et al., 2012). The mouse Reg proteins are classified into four types: (1) mReg1; (2) mReg2; (3) mReg3α, mReg3β, mReg3γ, and mReg3δ; and (4) mReg4. The human and rat Reg families are grouped into three types: (1) hReg1α, hReg1β, and rReg1; (2) hReg3α, rReg3β, hReg3γ, and rReg3γ; and (3) hReg4, rReg4 (Parikh et al., 2012) (TABLE A.).

Mouse	
Reg1	Reg, PSP, PTP (cleaved form), Lithostathine
Reg2	PTP2, PSP2, Lithostathine 2
Reg3α	PAP II
Reg3β	PAP I, PAP, HIP
Reg3γ	PAP III
Reg3δ	INGAP-rp, INGAP
Reg4	RELP

Rat	
Reg1	Reg, PSP, PTP (cleaved form), Lithostathine
Reg3α	Reg3, PAP II
Reg3β	PAP, PAP I, HIP, Reg2, Peptide23
Reg3γ	PAP III
Reg4	RegIV

Human	
REG1α	PSP, Lithostathine, PTP
REG1β	REGH, REGL, Lithostathine, RS
REG3β	REG3A, PAP, HIP, PAP I, Reg2, PTP
REG3γ	Reg3, PAP IB, PAP II, PAP III
REG4	REGIV

TABLE A. Classification of Reg members in (A) mouse, (B) human and (C) rat. Adopted from (Parikh et al., 2012).

All Reg family proteins contain a highly conserved C-type lectin domain (CTLD), which consists of six cysteine residues (Fig. 1.3). The six cysteine residues are located in identical positions, suggesting a similar organization of the disulphide bridges in the REG protein structures. CTLD enables REG proteins to protect the gut from intestinal microbiota (Lehotzky et al., 2010; Mukherjee et al., 2009; Mukherjee et al., 2013). The antibacterial effect results from their binding to peptidoglycan carbohydrates to recognize bacterial targets (Cash et al., 2006; Christa et al., 1994; Ho et al., 2010; Lehotzky et al., 2010), oligomerizing on the bacterial membrane and forming a membrane-penetrating pore (Mukherjee et al., 2013). Besides CTLD, there are some other important residues or motifs conserved in some of the REG proteins. The residues with dark green background (Fig. 1.3) are the prosegment of rREG1, mREG3γ, and hREG3α that are involved in bactericidal effect and fibril formation (Gurr W, 2012). The EPN motif (lime green background) and the loop1 and loop2 (grey background) in hREG3α (Fig. 1.3) were found to be involved in carbohydrate binding (Gurr W, 2012). Since the EPN motif is conserved in mREG3β, rREG3β, mREG3γ, rREG3γ, and hREG3α, as shown in Figure 1.3, it is logical to predict that these REG3 proteins might be also engaged in carbohydrate binding via specific amino acid residues. The N-terminal fragment (NtfrII, light blue background) and C-terminal fragment (CtfrII, lime green background) of mREG2 exhibited opposite roles in the development of T1DM in NOD mice (Gurr et al., 2007) (Fig. 1.3). Vaccination of NtfrII in NOD mice accelerated T1DM with activation of autoaggressive T-cells, while vaccination of CtfrII in NOD mice delayed T1DM (Gurr et al., 2007).

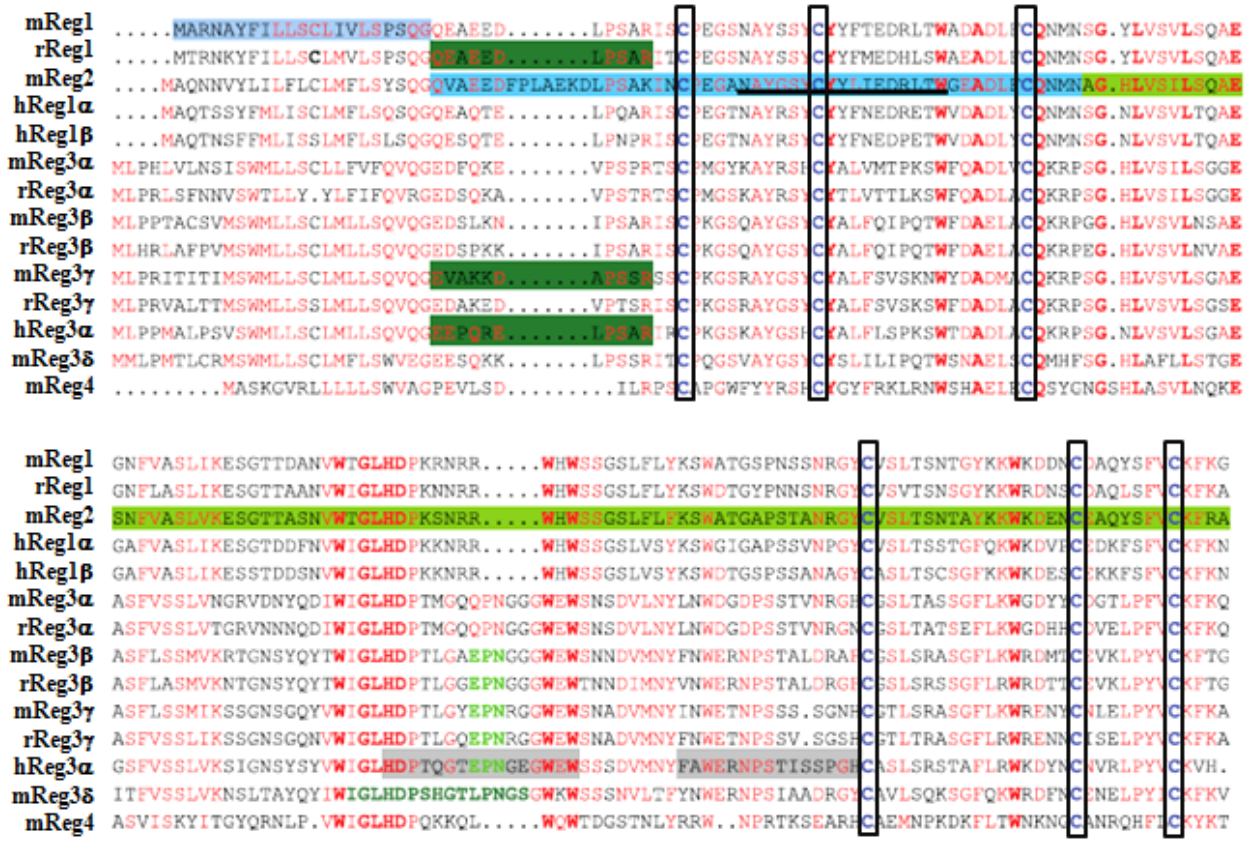


FIGURE 1.3 Primary sequence alignments for REG proteins. Adopted from (Gurr W., 2012).

The factors that regulate Reg expression include cytokines, hormones, and nutrients. Tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ) or a combination of interleukin-6 (IL-6) with dexamethasone treatment significantly induced *Reg1* mRNA expression in pancreatic acinar cells (Dusetti et al., 1996) and RINm5F β cells (Akiyama et al., 2001). Glucocorticoids directly stimulated the transcription of mouse *Reg2* and *Reg3β* genes in both pancreatic acinar and β cells (Luo et al., 2013). Interleukin-22 (IL-22) and interleukin-10 (IL-10) activated the mouse *Reg3β* mRNA transcription in acinar and AR42J cells, respectively. IL-6 activated rat *Reg3β* in PC12 cells (Broekaert et al., 2002), whose promoter region has two IL-6 response elements (T-

^{266}TCCCAG^{-260} and T^{-249}TCCCAG^{-243}) (Dusetti et al., 1995). The transcription factors activator protein-1 (AP-1), NeuroD1/Beta2, and signal transducer and activator of transcription (STAT) up-regulated the promoter activity of mouse *Reg3δ*, while PDX-1 suppressed the activity in HIT-T15 cells (Taylor-Fishwick et al., 2006).

The Reg family is a group of secretory proteins and was proposed to interact with its own putative receptors at the plasma membrane of the cells. Some research groups showed that Reg proteins were detectable in blood (Bacon et al., 2012; Carrère et al., 1999; Keel et al., 2009; Orelle et al., 1992; Zenilman et al., 2000) and might act as a hormone-like secretory substance with an endocrine or paracrine function (Carrère et al., 1999). Higher levels of circulating hReg1α were found in hepatocyte nuclear factor 1 alpha (HNF1A-MODY) patients and T1DM patients than in healthy individuals, suggesting hReg1α may be a clinical indicator of β cell apoptosis (Bacon et al., 2012). hReg1α was significantly increased in the serum of patients with sepsis and infections, indicating that it could be a marker for posttraumatic complications (Keel et al., 2009). Serum Reg1 and Reg3 levels are sensitive markers for pancreatic injury, as a large amount of the proteins was detected in the serum of pancreatitis patients and animal models (Orelle et al., 1992; Zenilman et al., 2000). Therefore, Reg proteins may serve as useful clinical biomarkers for the evaluation of exocrine pancreatic function, β cell apoptosis, acute pancreatitis, and other posttraumatic complications.

Reg proteins act as growth factors, acute phase reactants, lectins, and antimicrobial factors in islet β cells, pancreatic acinar cells, hepatocytes, neurons, enterocytes, and epithelial cells (Baeza et al., 1996; Cavard et al., 2006; Harris et al., 2012; Lai et al., 2012; Orelle et al., 1992). They are

involved in diseases such as diabetes (Gurr et al., 2007; Planas et al., 2006; Watanabe et al., 1994; Xiong et al., 2011), gastrointestinal cancer (Bishnupuri et al., 2006), hepatocellular carcinoma (Cavard et al., 2006; Christa et al., 1994), melanoma (Valery et al., 2001), neurodegeneration diseases (Acquatella-Tran Van Ba et al., 2012), and wound repair and psoriasis (Lai et al., 2012). In diabetes, Reg proteins exhibit different functions in various conditions. Reg1 and islet neogenesis-associated protein (INGAP-related peptide, Reg3) have been implicated in promoting of insulin synthesis and secretion via β cell proliferation/regeneration and neogenesis (Kakita et al., 2002; Rafaeloff et al., 1997; Takasawa et al., 2006; Terazono et al., 1988; Wise et al., 2004). Two-months intraperitoneal injection of REG1 protein (1mg/kg per day) in rats with surgical diabetes ameliorated their diabetes symptoms: decreased blood glucose with markedly decreased glucosurea, increased beta-cell mass, and elevated insulin secretion (Watanabe et al., 1994). However, contrary to the above discovery, mice overexpressing Reg1 in pancreatic α cell exhibited increased β cell apoptosis and decreased insulin secretion (Kondo et al., 2000). Mice overexpressing Reg3β specifically in pancreatic islets showed reduced insulin content in the pancreas, decreased islet GLUT2 protein, impaired glucose tolerance, and a decreased trend of GSIS both *in vivo* and *in vitro* (Xiong et al., 2011).

1.7 Regenerating Islet-Derived 2 (Reg2)

Reg2 was first discovered by Unno and co-workers in 1993 (Unno et al., 1993). It is a small protein with 173 amino acids (Fig. 1.4A). Mouse REG1 and REG2 share 76% amino acid homology (Unno et al., 1993), which helps to predict the three-dimensional protein structure of REG2 (Fig. 1.4B) based on the crystal structure for REG1 (Bertrand et al., 1996). However, they

possess several unique characteristics, and their regulations and functions vary (Baeza et al., 1997; Deane et al., 2002; Lasserre et al., 1994; Milner and Hales, 1967; Perfetti et al., 1996; Planas et al., 2006; Unno et al., 1993; Zhong et al., 2007).

Several studies have indirectly implied that REG2 was a stress-induced protein. Both Reg2 messenger RNA and protein level were significantly increased by chemical-induced pancreatitis in mice (Huszarik et al., 2010; Lu et al., 2006; Zhong et al., 2007). *Reg2* gene promoter activities in pancreatic acinar and islet cells were also significantly increased by glucocorticoids and IL-6 treatment (Luo et al., 2013), which suggested that the *Reg2* gene promoter was highly responsive to pancreatic inflammation and oxidative stress. AP-1 is an oxidative stress sensor transcription factor. The AP-1 binding sites in 1500 bp Reg2 promoter region (Fig. 1.4C) may provide a link between an oxidative stress and REG2 transcriptional regulation.

The biological function of Reg2 in T2DM remains elusive. Reg2 was found to be expressed in pancreatic β cells (Gurr et al., 2007; Huszarik et al., 2010; Planas et al., 2006), acinar cells (Wang et al., 2011c; Zhong et al., 2007), or both (Lu et al., 2006). However, Reg2 seems to be more functional in β cells than in acinar cells. Overexpression of the *Reg2* gene in mouse insulinoma cells protected the cells from STZ-induced mitochondrial apoptosis (Liu et al., 2010), while pancreatic acinar-specific overexpression of Reg2 in mice did not change glucose homeostasis nor show any protection against diabetes and pancreatitis (Li et al., 2010). REG2

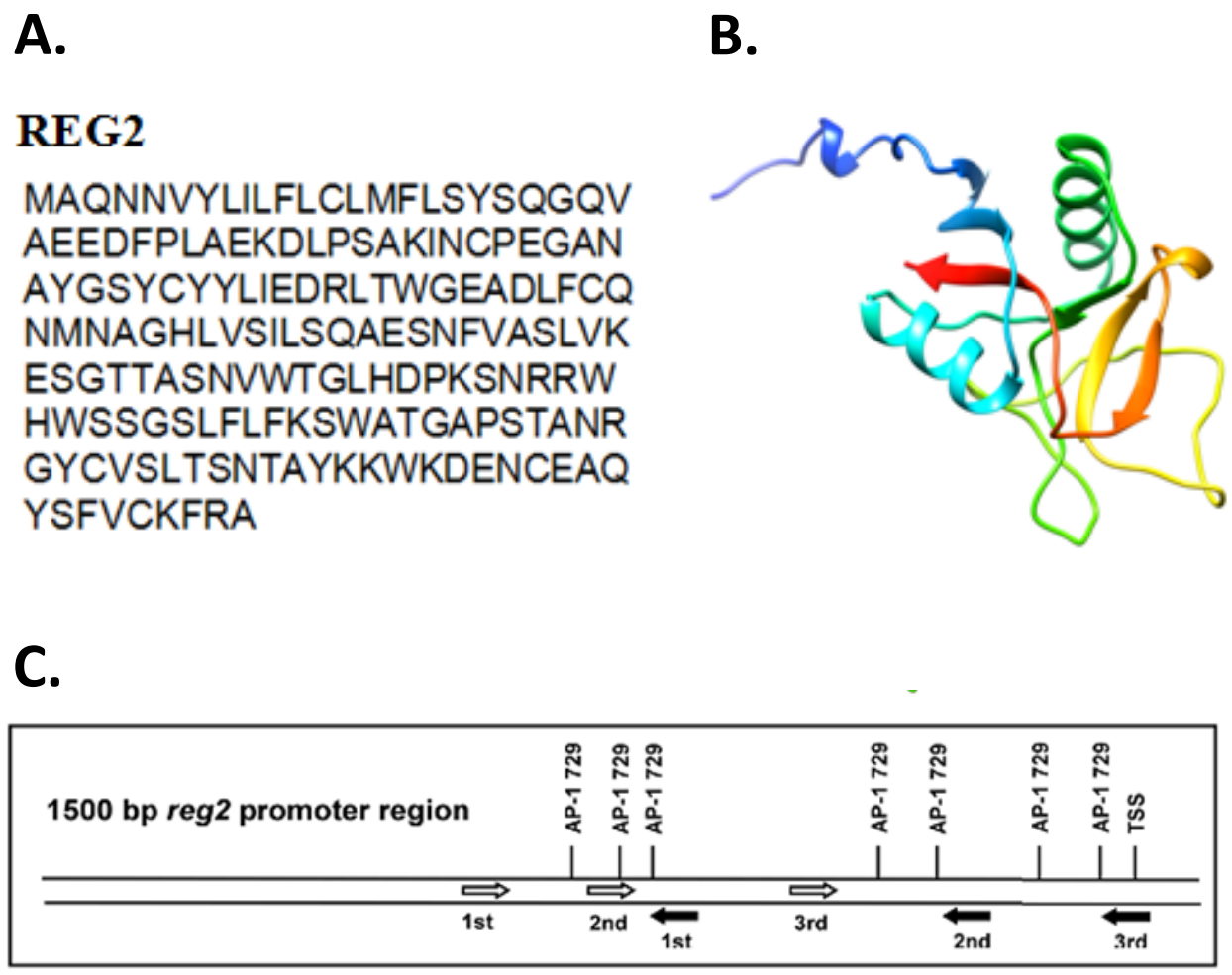


FIGURE 1.4 Bioinformatics analysis of mouse Reg2. (A) Amino acid sequence. (B) Predicted three dimensional protein structure. (C) 1500 bp *Reg2* promoter region with putative AP-1 binding sites.

overexpression in NOD diabetic islets correlated with the acceleration of diabetes (Planas et al., 2006). Also, REG2 was reported to be a novel β cell-derived autoantigen in NOD mice that may be involved in islet self-destruction (Gurr et al., 2007).

To identify the underlying molecular mechanism for the hyperinsulinemia in OE mice, we screened 25 insulin-related gene changes in WT and OE islets. We found that, when compared to WT islets, OE islets exhibited higher insulin-related gene expression, such as *Ins1, Pdx1* and *Foxa2,* and lower *Ucp2* expression (Fig. 1.5A). More strikingly, we observed that *Reg2* mRNA expression was diminished in OE islets when compared with WT islets across all ages (Fig. 1.5A, B). However, no differences in the expression of *Reg1* were found in the islets of the two genotypes (Fig. 1.5A).

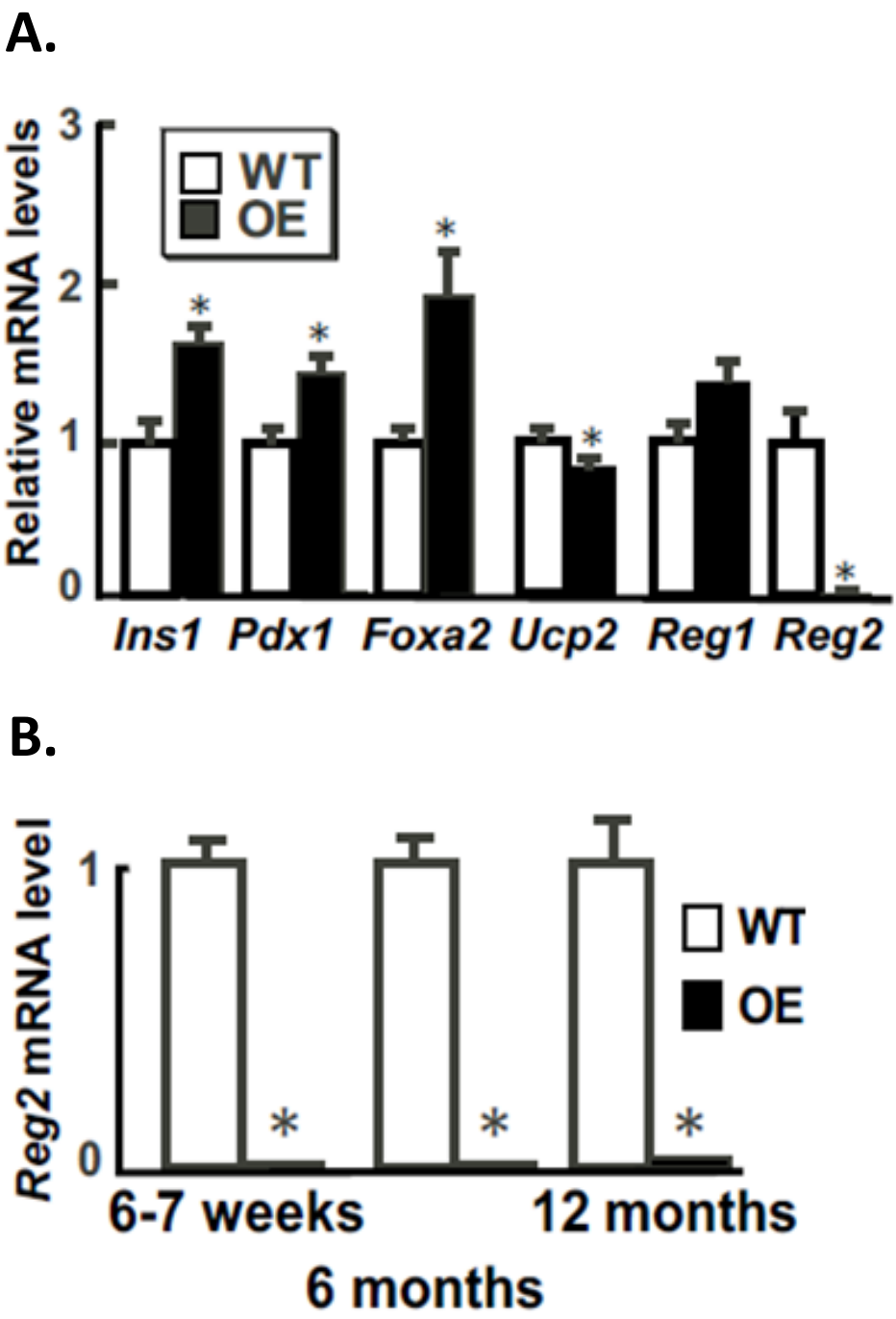


FIGURE 1.5 Different insulin-related gene expressions in WT and OE islets. (A) mRNA level of *Ins1*, *Pdx1*, *Foxa2*, *Ucp2*, *Reg1* and *Reg2* in WT and OE islets. (B) *Reg2* mRNA level in WT and OE islets isolated from 6-7 weeks, 6 months and 12 months old mice. Data presented as mean ± SE (*n* = 5 to 6 for panels A and B). Means for a given gene without sharing a common letter differ (* $P < 0.05$ vs. WT).

1.8 Objectives

Our lab previously showed T2DM-like phenotypes in mice overexpressing GPX1 (McClung et al., 2004). And we demonstrated the hyperinsulinemia phenotype as the primary outcome of GPX1 overproduction (Wang et al., 2008). Thus, the overall objective of this dissertation is to understand how GPX1 overexpression led to the T2DM-like phenotypes. Because we identified an islet gene, *Reg2,* whose mRNA level in islets was diminished by GPX1 overproduction (Fig. 1.5A, B), we hypothesized that the depleted REG2 in OE islets mediated the OE T2DM-like phenotypes. In Chapter 2, the objectives were to determine whether or not ROS regulates REG2 protein expression and whether or not exogenous REG2 administration alleviates T2DM-like phenotypes in OE mice. In Chapter 3, the study was designed to investigate the molecular mechanisms of REG2 protein in regulating insulin secretion *in vitro*.

Although Se exhibited anti-diabetic effects (Faure, 2003; Mueller and Pallauf, 2006), an accumulating number of recent human studies have shown that Se supplements had hyperglycemic, hyperlipidemic, and pro-diabetic effects (Bleys et al., 2007; Bleys et al., 2008; Laclaustra et al., 2010; Stranges et al., 2010a; Stranges et al., 2007; Stranges et al., 2010b). Because GPX1 is the most abundant selenoprotein in the body, OE mice serve as an ideal animal model to study how Se supplements potentiate T2DM in humans. In Chapter 4, the research was focused on whether or not dietary Se deficiency precluded GPX1 overproduction and rescued T2DM-like phenotypes in OE mice.

CHAPTER 2

Metabolic Function of REG2 in GPX1 Overexpressing Mice

2.1 Abstract

Previously, we found that cellular GPX1 overexpressing (OE) mice developed hyperglycemia, hyperinsulinemia, insulin resistance and obesity, and that hyperinsulinemia and hyper-secretion of insulin after glucose stimulation were primary outcomes of GPX1 overexpression in OE mice. More strikingly, we found that OE islets had depleted *Reg2* mRNA compared with WT islets. In order to determine how REG2 is downregulated by GPX1 overexpression and whether REG2 is the mediator of the T2DM-like phenotypes of OE mice, we examined the REG2 protein change after treating islets with various chemicals that could alter intracellular ROS level, and we examined the change of metabolic parameters after injecting 1-year-old OE mice with recombinant REG2 protein. We found that the expression of islets REG2 protein level dramatically decreased in OE as compared to the WT mice. We showed that ebselen, sodium selenite (Na_2SeO_3), or *N*-acetyl cysteine (NAC) treatment significantly downregulated REG2 level, while H_2O_2 treatment up-regulated REG2 level in WT and OE islets. Diquat (DQ) or streptozotocin (STZ) injection in mice also increased REG2 protein level in both WT and OE pancreas. Fifteen-minute pre-injection of recombinant REG2 protein significantly decreased glucose stimulated insulin secretion (GSIS) and glucose tolerance in OE mice, and two-week long daily administration of exogenous REG2 protein alleviated hyperinsulinemia, hyperglycemia and hypertriglyceridemia in 1-year-old OE mice. Thus, GPX1 down-regulated REG2 level by modulating intracellular ROS level in islets, while exogenous REG2 administration partially rescued T2DM-like phenotypes in OE mice.

2.2 Introduction

GPX1 is a major intracellular antioxidant enzyme functioning as a scavenger of intracellular ROS by using GSH as a substrate (de Haan et al., 1998; Esposito et al., 2000; Mirochnitchenko et al., 1995; Taylor et al., 1993). Although some studies demonstrated the protective role of GPX1 in oxidative injuries (Cheng et al., 1999a; Crack et al., 2001; Fu et al., 2001), accumulating evidence suggests that dysregulation of GPX1 is involved in the development of metabolic syndrome. An up-regulation of GPX1 activity has been correlated with higher insulin levels and insulin resistance in healthy pregnant women (Chen et al., 2003). OE mice developed hyperinsulinemia, elevated GSIS, hyperglycemia, insulin resistance, and obesity at the age of six months (McClung et al., 2004), while chronic hyperinsulinemia was defined as a primary outcome of GPX1 overproduction in OE islets (Wang et al., 2008). Compared with wild type (WT), OE islets showed a 20-fold enhancement of GPX1 activity, decreased intracellular ROS production, and increased mitochondrial membrane potential (Wang et al., 2008).

Islet *Reg2* mRNA was diminished in the OE mice aged 6-7 weeks, 6 months, and 12 months (Fig. 1.5B). Pancreatic REG2 protein level was found to be inversely related to GPX1 expression in mice fed a high-fat diet (Qiu et al., 2005). Oxidative stress might mediate the inverse correlation between Reg2 and GPX1 as several studies have implied that REG2 is a stress-induced protein. *Reg2* gene promoter activity in both pancreatic acinar and islet cells was significantly increased by glucocorticoids treatment (Luo et al., 2013), which helps explain the up-regulated mRNA and protein expression of Reg2 in mice with chemical-induced pancreatitis (Huszarik et al., 2010; Lu et al., 2006; Zhong et al., 2007).

Reg2 was shown to be inversely correlated with low insulin levels. NOD diabetic mice had increased pancreatic *Reg2* expression while no detectable pancreatic *insulin* mRNA (Sanchez et al., 2000). Overexpression of *Reg2* in islets correlated with acceleration of diabetes in the NOD mouse model (Planas et al., 2006; Sanchez et al., 2000). Human Reg1β (hReg1β), which shares 63% homology with mouse Reg2 (Thompson et al., 2002), was found to have a higher expression in adult than in fetus pancreas, while *insulin* gene expression showed the opposite change to *hReg1β* (Sanchez et al., 2001).

Considering the diminished islet *Reg2* mRNA in OE mice, and the correlation among Reg2, oxidative stress and diabetes, we raised the questions of how REG2 expression was down-regulated by GPX1 overexpression and whether or not the depleted REG2 was the mediator of the T2DM-like phenotypes of OE mice. To address the questions, we examined the REG2 protein change after treating islets with various chemicals that can alter intracellular ROS level, and we determined metabolic responses of the 1-year-old OE mice after injections of the recombinant REG2 protein.

2.3 Materials and Methods

Mouse and Diet

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Cornell University. Mice were reared in plastic cages in an animal room with a constant temperature (22°C) and a 12-h light:dark cycle, and were given a Torula yeast and sucrose-based diet (TABLE B.) (Cheng et al., 1997) with 0.3 mg Se/kg (as sodium selenite) and distilled water added. The OE mice were derived from a B6C3 (C57B1 x C3H) hybrid line (Cheng et al., 1997).

Mice were feed-deprived for 8 hours overnight before measurements, tests, and tissue sample collection.

Quantitative Real Time PCR Analysis

Islets were isolated from WT and OE mice using collagenase and were handpicked under a dissection microscope, as outlined in the standard protocol with minor modifications (Wang et al., 2008). Total RNA was extracted from the primary islets by using the Trizol reagent (Invitrogen, Carisbad, CA). The concentration and integrity of diluted samples were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and 1 μg (10 μl) of RNA was reverse transcribed into cDNA using 1 μl each of dNTP and Oligo(dT) in a 5 minute 65°C reaction using a thermocycler, followed by 4 μl 5x First Strand Buffer, 2 μl 0.1 M DTT, 1 μl RNase OUT, and 1 μl Superscript III at 50°C for 50 min and 70°C for 15 minutes. 1 μl of cDNA was loaded into each well of 384 well qPCR plates and was reacted with 3 μl ddH_2O, 5 μl SybrGreen PCR Master Mix and 0.5 μl forward and 0.5 μl reverse primer for 40 cycles with a Tm of 60°C. *β-actin* was used as a reference gene and the $2^{\wedge -ddCt}$ method was used for all analysis of the results (Schmittgen and Livak, 2008).

Immunoblotting

Islet, pancreas, or other tissues were placed in a protein lysis buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na_3VO_4, 100 mM NaF, 50 mM $Na_4P_2O_7$, 10 mM EGTA, 10 mM EDTA, 1% Triton X-100, 5 μg/ml aprotinin, 10 nM okadaic acid, and 2 mM phenylmethylsulfonyl fluoride (PMSF). After homogenization on ice, cell lysate was centrifuged at 4°C, 14000RPM, for 15 minutes. The supernatant was collected and assayed for protein quantification via BCA assay

(Thermo Scientific, Rockford, IL). The rest supernatant was used for Immunoblotting analysis. For Immunoblotting, islet lysate supernatant was heated at 100°C heat block for 5 minutes and resolved by an SDS-PAGE gel, transferred to a nitrocellulose membrane, and blocked in a 3% milk/TBST solution for 1 hour. The membrane was incubated with primary antibodies (diluted in TBST) at 4°C, overnight, washed in TBST (3 X 15 minutes), and incubated with secondary antibodies (also diluted in TBST) for 1 hour at room temperature. Following 3X washes in TBST, the signals on the membrane were detected using a Pierce ECL Immunoblottingting System (Thermo Scientific, Rockford, IL).

Immunohistochemistry

The whole pancreatic tissue was removed from WT mice, fixed in 4% paraformaldehyde (PFA) at 4°C overnight, washed 3 times in PBS, and transferred into 70% ethanol and placed into a cassette for embedding (Histology Lab, Schurman Hall, Cornell University). The embedded samples were sectioned at 5 μm, and the slides were rehydrated through xylenes and decreasing grades of alcohol. The slides were then washed with PBS/Triton X-100, blocked with 10% BSA/PBS for 1.5 hours, incubated with REG2 primary antibody (diluted 1:500 in 3% milk/PBS) overnight at 4°C. The next day, the slides were washed with PBS/Triton X-100, and secondary antibody was applied (diluted 1:2000 in 1% BSA/PBS) at room temperature for 1 hour. After washing with PBS/Triton X-100, the slides were dehydrated through increasing grades of alcohol and xylenes. Positive staining was observed via fluorescence microscopy. Carol A. Roneker performed this procedure.

Immunoprecipitation

A whole cell lysate protein was incubated with protein G beads (Santa Cruz, Dallas, TX) with/without REG2 antibody (a gift from Dr. Omary) (Zhong et al., 2007) at 4°C overnight. Protein G beads with REG2 antibody served as the negative control. Samples were washed five times with washing buffer, and proteins were eluted using an SDS-PAGE loading buffer at 100°C for 5 minutes. After centrifugation, supernatant was loaded into SDS-PAGE gels for immunoblotting detection.

Islet Culture

After being isolated from the pancreas, the islets were recovered for 2 hours in RPMI 1640 medium (Gibco, Grand Island, NY) with 10% fetal bovine serum, 2 mM glutamine, 11.1 mM glucose, 1% PenStrep, and incubated at 37°C in a 5% CO_2 humidified incubator. Different doses of ebselen, Na_2SeO_3, NAC, or H_2O_2 were added into the culture medium, and islets were treated for six or twelve hours. Islets were then washed with HBSS buffer three times before transferred to protein lysis buffer for immunoblotting analysis.

Drug Administration

WT and OE mice were fasted overnight for eight hours before each administration. DQ was prepared in phosphate buffer, while STZ (Sigma-Aldrich, St. Louis, MO) was prepared in citrate buffer solution immediately before intraperitoneal (i.p.) administration. Mice were i.p. injected with DQ (24 μg per g body weight), STZ (150 μg per g of body weight), or saline (10 μl per g of body weight). After 48h, the mice were euthanized by CO_2 inhalation. Plasma and pancreas were collected and plasma glucose, pancreatic REG2 level, and pancreas morphology were estimated.

REG2 Expression and Purification

The NOD mouse *Reg2* gene (G22 to A173) was expressed with an N-terminal hexahistidine tag in *Escherichia coli* using a pQE-30 series vector (Qiagen, Valencia, CA). It is a gift from Dr. Werner Gurr (Gurr et al., 2007). Cells grown in LB media (100 µg/mL ampicillin, 25 µg/mL kanamycin) were induced with 1 mM IPTG, then lysed by sonication in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0). The clarified lysate was passed over an Ni-NTA agarose (Qiagen, Valencia, CA) column, washed with lysis buffer at pH 6.3, and REG2 eluted as a single peak with lysis buffer at pH 3.8. The affinity purified REG2 was further purified by ion-exchange chromatography using Macro-Prep high S (Bio-Rad, Hercules, CA) using a linear gradient from 0 to 1 M NaCl in 50 mM Na-acetate, 8 M urea, pH 5.0. REG2 was dialyzed against 50 mM Na-acetate, 4 M urea, pH 5.0 for studies.

The same *Reg2* gene was also expressed in the native form (without a hexahistidine tag) in the yeast *Pichia pastoris* GS200. To express REG2 without non-native amino acids at the *N*-terminius, the gene was cloned flush with the Kex2 cleavage site of a modified alpha-factor secretion signal peptide in the pPIC9 vector (Invitrogen, Carisbad, CA), and transformed into yeast using electroporation (BTX, Holliston, MA). After induction in BMMY for 4.5 days, the clarified supernatant was brought to 60% saturation with ammonium sulfate to precipitate the REG2. The precipitate was dissolved in 50 mM Na-acetate, pH 4.0, then dialyzed against the same buffer, after which REG2 was precipitated. The precipitate was dissolved in 50 mM Tris-HCl, pH 7.6, then subjected to ion-exchange chromatography using Macro-Prep high Q (Bio-Rad, Hercules, CA) using a 0 to 1 M NaCl linear gradient in the same buffer. The REG2

containing fractions were pooled, concentrated by ammonium sulfate precipitation, then dissolved in and dialyzed against PBS, pH 7.4.

Recombinant REG2 Protein Administration

Both REG2 proteins were filter sterilized using a 0.2 μm syringe filter. The protein concentrations were measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL), after which REG2 was frozen in aliquots at -80 ℃. For a short-term study, 2-6 μg/g REG2 protein was i.p. administrated into WT or OE mice. After 15 minutes, glucose tolerance test (GTT) and GSIS tests were performed. For a long-term study, 2-6 μg/g REG2 protein was daily i.p. injected into 1-year-old OE mice for 2 weeks. Fasting body weight, blood glucose, plasma insulin and plasma triglyceride (TG) were measured every 7 days. GSIS, GTT, and insulin tolerance test (ITT) were performed before and after 14 days' injection.

GTT, GSIS, ITT and TG Measurements

For the GTT, after an overnight fasting (approximately 8 hours), mice were i.p. administrated with a sterile solution of glucose at a final concentration of 1 g/kg body weight. Blood glucose levels were measured from blood drops of each mouse tail at 0, 15, 30, 60 and 120-minute time points using the glucometer and accompanying strips (Bayor Contour, Tarrytown, NY). For the GSIS, a capillary tube of blood was taken from each mouse following glucose challenge at the 0, 15, 30 and 60-minute time points. The blood samples were transferred into individual centrifuge tubes and then centrifuged at 4°C at 2000 RPM for 5 minutes. The clear plasma layer was collected and measured for insulin by using a rat/mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL). For the ITT, the fasted mice were i.p. injected with a sterile solution of

insulin (Eli Lily, Indiannopalis, IL) at a final concentration of 1 U/kg body weight. Blood glucose was measured from blood drops of each mouse tail by using glucometer and accompanying strips (Bayor Contour, Tarrytown, NY), following insulin challenge at the 0, 30, 60, 120, 180 and 240-minute time points. For TG measurement, the fasted tail blood samples were collected and transferred into individual centrifuge tubes and then centrifuged at 4°C at 2000 RPM for 5 minutes. The clear plasma layer was collected and measured for the concentration of TG using a TG kit (Wako Chemicals, Richmond, VA).

Statistical Analysis

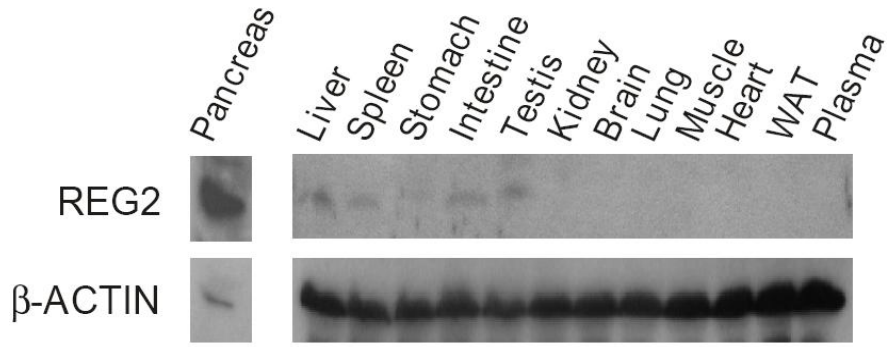
Values were expressed as mean ± SE. Significance was defined as $P < 0.05$. The most of statistical comparisons between each group were performed by using one way ANOVA. In some experiments Dunnett's test was used. Statistics was calculated using SAS 6.11 software (SAS Institute, Cary, NC).

2.4 Results

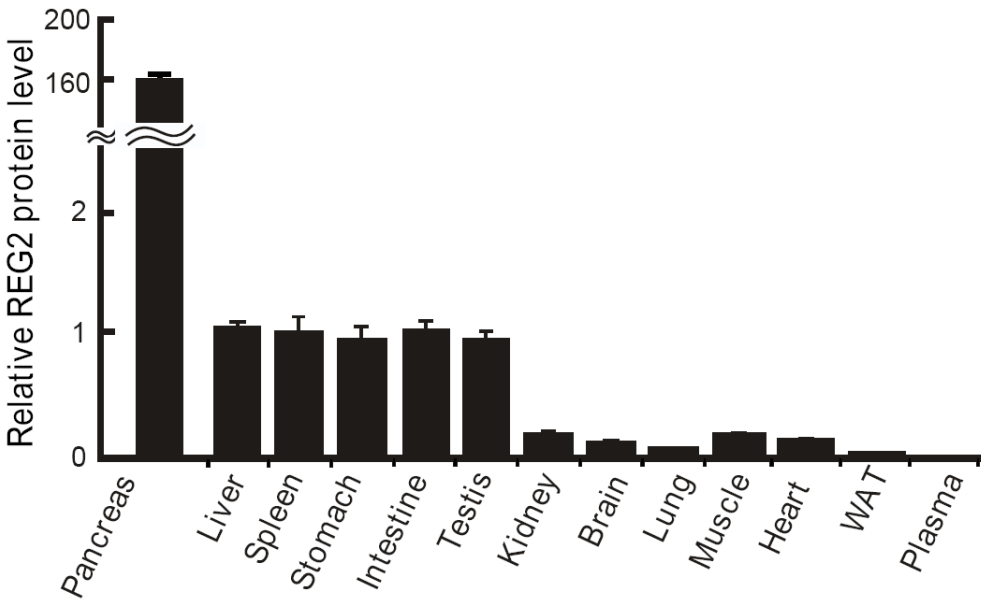
2.4.1 REG2 Was Mainly Expressed in Pancreas and Was Depleted in OE Islets

To study the tissue distribution of REG2 protein in 6-month-old WT male mice, we collected fresh tissues from the mice and measured the REG2 protein level with the immunoblotting method. Strikingly, we observed that the REG2 level was 160 times higher in the pancreas than in the liver ($P < 0.05$), while REG2 levels in the spleen, stomach, intestine, and testis were similar to those in the liver (Fig. 2.1A, B). The kidney, brain, lung, muscle, and heart showed 16.5%, 9.4%, 5.1%, 17.9%, and 13.3% of REG2 level, respectively ($P < 0.05$), when compared

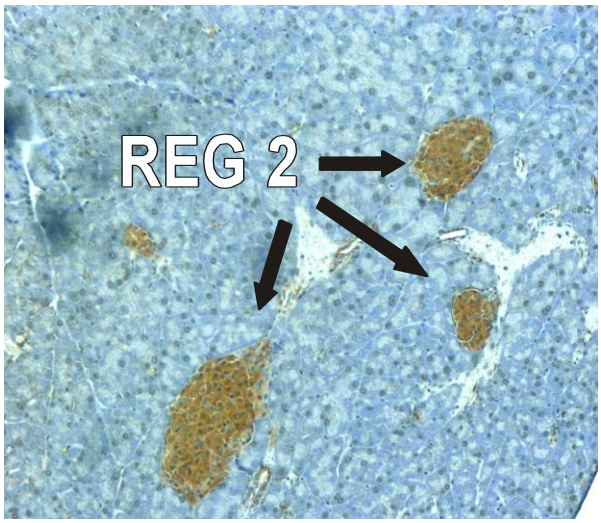
A.


Pancreas
Liver
Spleen
Stomach
Intestine
Testis
Kidney
Brain
Lung
Muscle
Heart
WAT
Plasma
REG2
β-ACTIN

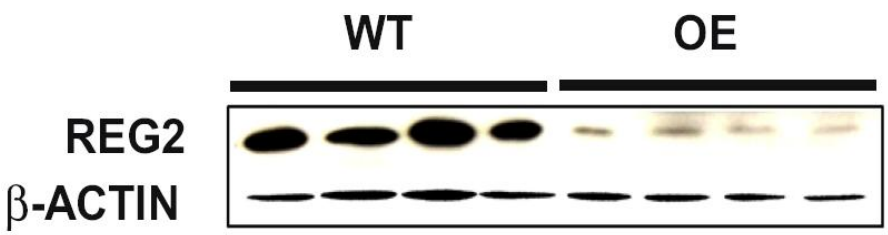
B.


Relative REG2 protein level
200
160
2
1
0
Pancreas
Liver
Spleen
Stomach
Intestine
Testis
Kidney
Brain
Lung
Muscle
Heart
WAT
Plasma

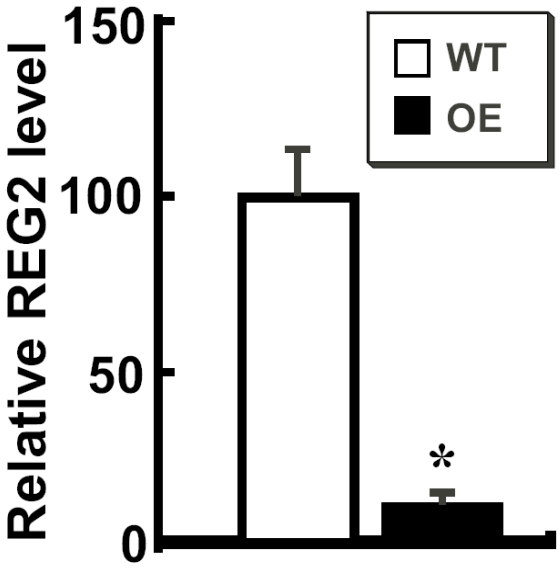
C.


REG 2

D.


WT
OE
REG2
β-ACTIN

E.


150
100
50
0
Relative REG2 level
WT
OE
*

F.

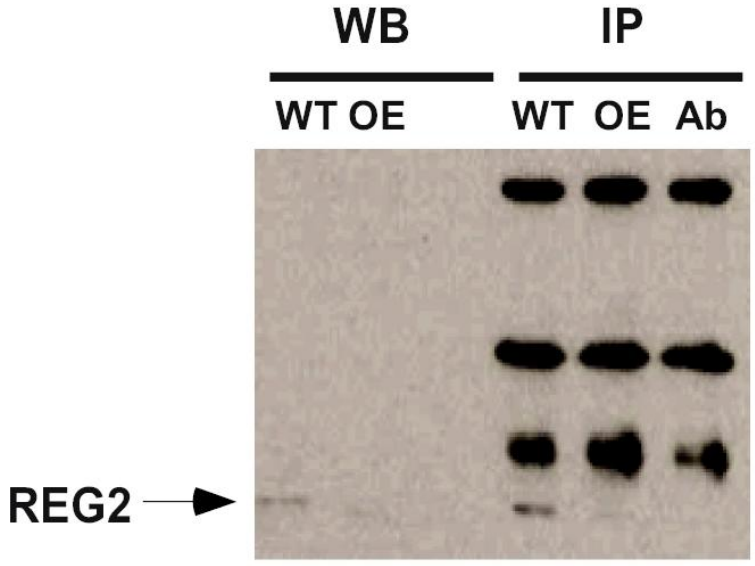

WB
IP
WT OE
WT OE Ab
REG2

FIGURE 2.1 REG2 protein level in different tissues and islets.

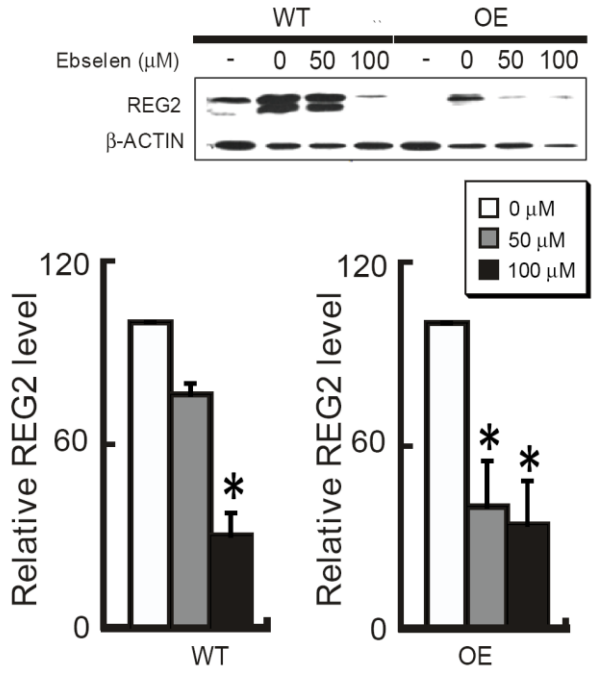
(A, B) Immunoblotting analysis of REG2 protein level in different tissues of WT mice, and the quantification of the band densities by Image J software. (C) REG2 immunostaining of islets (brown color) in representative pancreatic sections from a WT mouse. (D, E) Immunoblotting analysis of REG2 level in WT and OE islets, and the quantification of the band densities by Image J software. (F) Immunoprecipitation of endogenous REG2 from WT and OE pancreas, and Immunoblotting analysis of REG2 protein. Data presented as mean ± SE (n = 3 to 4 for panels B, n=4 for panels E). Means for a given protein without sharing a common letter differ (* $P < 0.05$ vs. WT).

to the liver (Fig. 2.1A, B). The REG2 levels in white adipose tissue and plasma were too low to be detected by the immunoblotting approach (Fig. 2.1A, B). The immunohistochemistry analysis for the WT pancreas showed that REG2 was mainly expressed in the islets rather than in the exocrine pancreas (Fig. 2.1C). As we observed diminished *Reg2* mRNA levels in OE islets spanning different ages (Fig. 1.5B), we used immunoblotting and immunoprecipitation approaches to compare the endogenous REG2 protein expression in WT and OE islets. In the comparison with WT islets, we confirmed the depleted endogenous REG2 protein level in OE islets (Fig. 2.1D, E). We also observed that the endogenous pancreatic REG2 protein level pulled down by REG2 antibody was dramatically decreased in OE islets (Fig. 2.1F).

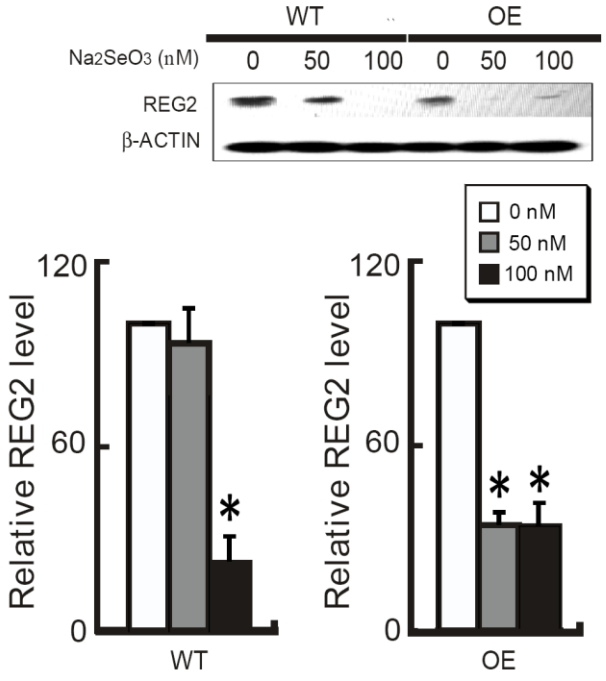
2.4.2 Reduced ROS Level Decreased REG2 Protein Production in Islets

Because Reg2 was implied as a stress-induced protein (Huszarik et al., 2010; Lu et al., 2006; Zhong et al., 2007), we hypothesize that oxidative stress serves as the mediator of the diminished REG2 by GPX1 overproduction in islets. To test this hypothesis, we examined the REG2 protein change in islets after treating them with various chemicals that can alter their intracellular ROS level. To decrease their intracellular ROS level, isolated islets were treated with antioxidants such as ebselen, Na_2SeO_3, or NAC, and the REG2 protein change was estimated at the end of the study. Ebselen serves as a GPX1 mimic and a potent scavenger of H_2O_2 (Müller et al., 1984). Na_2SeO_3 is an inorganic form of Se that can increase GPX1 activity. NAC enhances GSH synthesis to neutralize H_2O_2. After 12 hours of 100 μM ebselen, 100 nM Na_2SeO_3, or 6 mM NAC treatment, a significant decrease in REG2 levels was observed in both WT and OE islets (Fig. 2.2A, B, C). It was noteworthy that OE islets shoed more reduction in REG2 levels than WT islets after 50 μM ebselen, 50 nM Na_2SeO_3, or 3 mM NAC treatment (Fig. 2.2A, B, C).

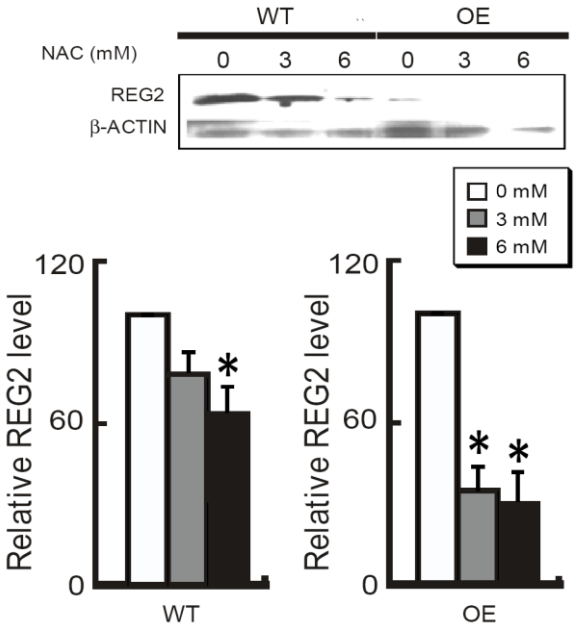
A.


WT
OE
Ebselen (μM)
- 0 50 100 - 0 50 100
REG2
β-ACTIN
0 μM
50 μM
100 μM
Relative REG2 level
120
60
0
*
*
WT
OE

B.


WT
OE
Na2SeO3 (nM)
0 50 100 0 50 100
REG2
β-ACTIN
0 nM
50 nM
100 nM
Relative REG2 level
120
60
0
*
*
WT
OE

C.


WT
OE
NAC (mM)
0 3 6 0 3 6
REG2
β-ACTIN
0 mM
3 mM
6 mM
Relative REG2 level
120
60
0
*
*
*
WT
OE

D.

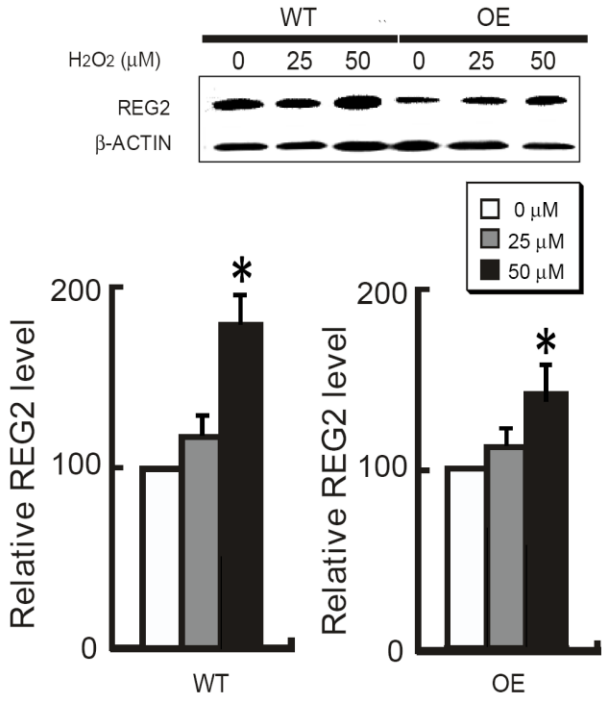

WT
OE
H2O2 (μM)
0 25 50 0 25 50
REG2
β-ACTIN
0 μM
25 μM
50 μM
Relative REG2 level
200
100
0
*
*
WT
OE

FIGURE 2.2 Effects of oxidative stress on islet REG2 protein production *in vitro*.

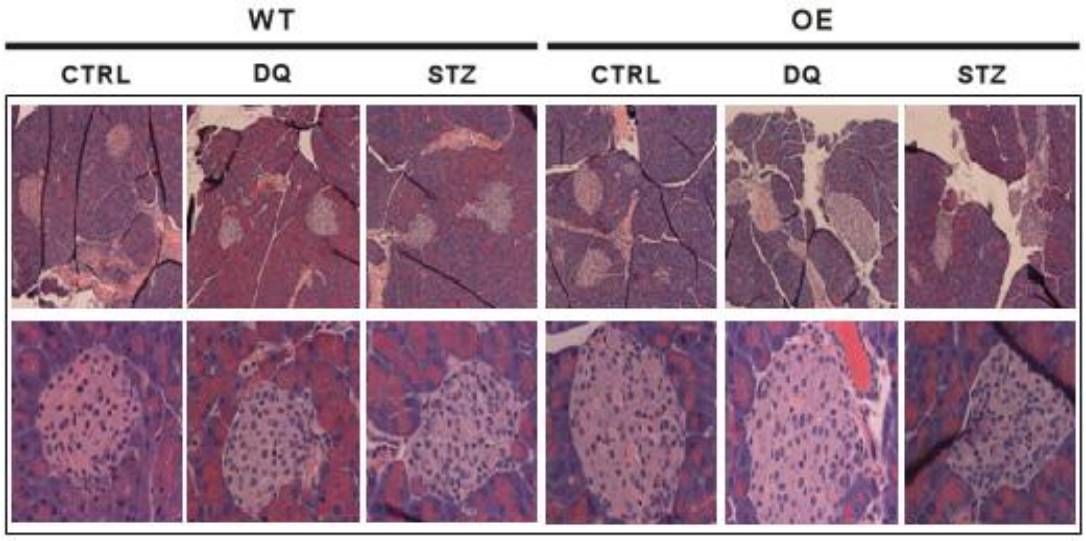
REG2 protein level in WT and OE islets after (A) 12 hours of 50 and 100 μM ebselen treatment; (B) 12 hours of 50 and 100 nM Na_2SeO_3 treatment; (C) 12 hours of 3 and 6 mM NAC treatment, and (D) 6 hours of 25 and 50 μM H_2O_2 treatment.. Data presented as mean ± SE (n = 10 to 11 for panels A, B and C, n = 5 for panels D). Means for a given measure without sharing a common letter differ (* $P < 0.05$ vs. control (CTRL)).

2.4.3 Increased ROS Level Induced REG2 Protein Production both *in Vitro* and *in Vivo*

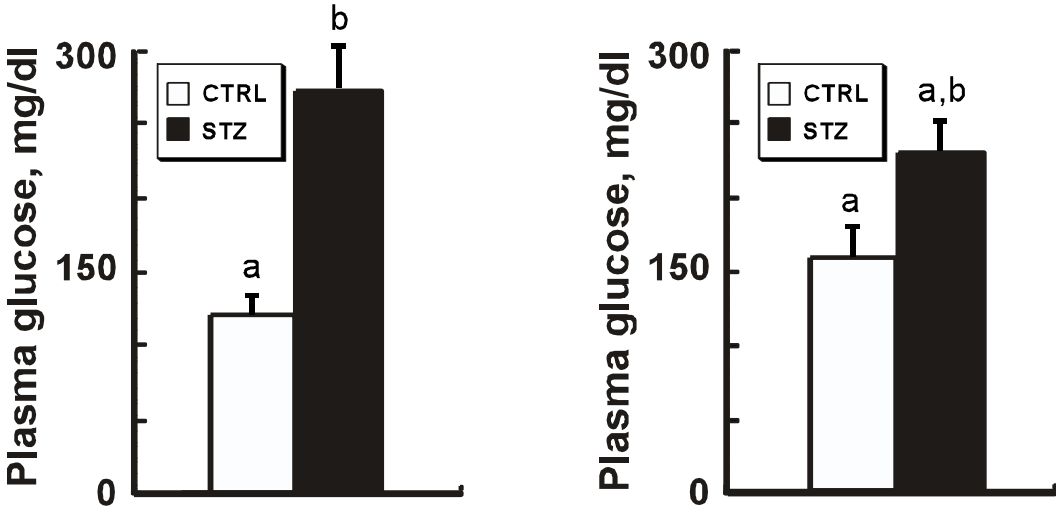
To increase intracellular ROS levels, oxidative stress inducers H_2O_2, DQ, and STZ were used to treat either islets or mice. H_2O_2 can diffuse across biological membranes and react with cellular components (Thannickal and Fanburg, 2000). DQ is an herbicide that is also associated with cellular production of ROS, including H_2O_2, O_2^-, and $^{\cdot}OH$ (Farrington et al., 1973; Stancliffe and Pirie, 1971). STZ is a glucosamine-nitrosourea compound that resembles glucose, as it is transported into the β cells by GLUT2 and damages their DNA fragmentation through H_2O_2 generation (Takasu et al., 1991; Wang and Gleichmann, 1998).

Firstly, islets were treated with 0, 25, or 50 μM H_2O_2 for 6 hours, and REG2 protein expression was determined. After 50 μM H_2O_2 treatment, WT and OE islets showed a 78.1% and 41.9% increase of REG2 protein level respectively ($P<0.05$) in comparison to their own control group, while there was no significant change after the 25 μM H_2O_2 treatment (Fig. 2.2D). Next, mice were injected with buffer, DQ, or STZ, and plasma glucose concentration, REG2 levels, and morphological changes of pancreas were estimated 48 hours after the injection. Both the WT and OE pancreas showed oedema with enlarged secretory granules and atrophic rough endoplasmic reticulum on the basal side, while the islet cells exhibited a slightly irregular shape, pycnosis and vacuoles, or vesiculation after DQ or STZ injection (Fig. 2.3A). Pancreatic endocrine damage was corroborated by the markedly decreased insulin level, which is reflected by elevated blood glucose (Dirice et al., 2011). After 48 hours of treatment, WT mice showed 123.6% higher plasma glucose ($P<0.05$) as compared with the control (Fig. 2.3B, left panel), but OE mice only

A.


WT
OE
CTRL
DQ
STZ
CTRL
DQ
STZ

B.


Plasma glucose, mg/dl
300
150
0
CTRL
STZ
a
b
Plasma glucose, mg/dl
300
150
0
CTRL
STZ
a
a,b

C.


WT
CTRL
DQ
STZ
REG2
β-ACTIN
OE
CTRL
DQ
STZ
REG2
β-ACTIN

D.

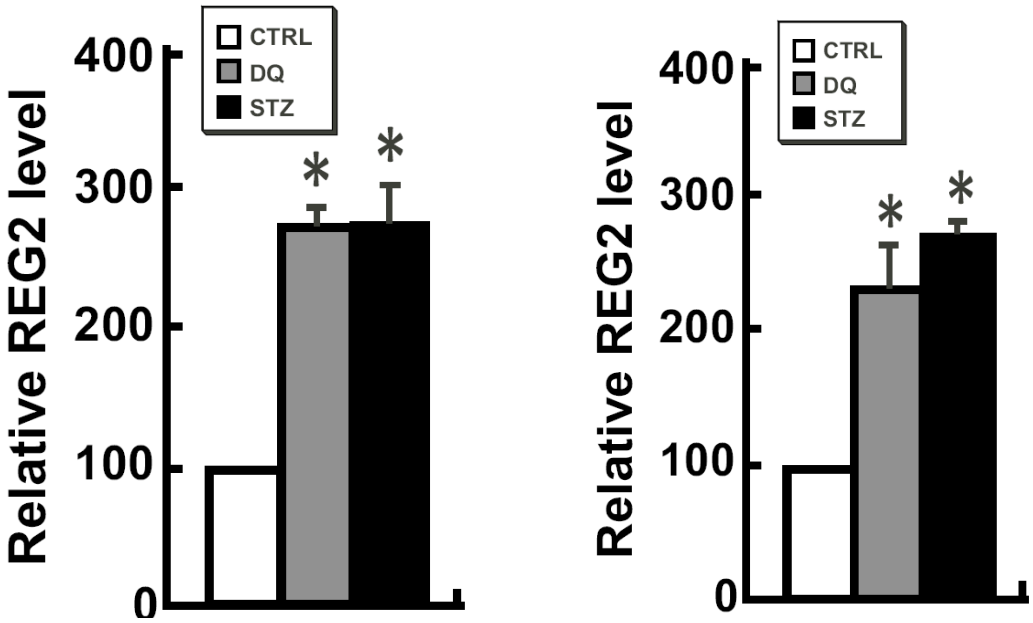

Relative REG2 level
400
300
200
100
0
CTRL
DQ
STZ
*
*
Relative REG2 level
400
300
200
100
0
CTRL
DQ
STZ
*
*

FIGURE 2.3 Effects of oxidative stress on pancreatic REG2 protein production *in vivo*.

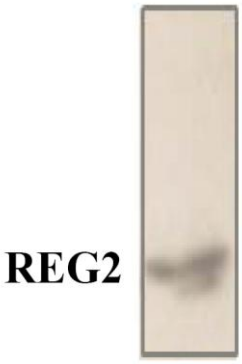
DQ and STZ were injected intraperitoneally into mice at concentrations of 24 mg and 150 mg per kg of body weight, respectively. Saline injection of 10 ul/ g of body weight served as control. After 48 hours, mice were sacrificed and plasma and pancreas were collected. (A) H&E staining of pancreas sections from WT and OE mice (10X magnification). (B) Plasma glucose level in WT and OE mice. (C, D) REG2 protein level in the pancreas was analyzed by using Western blot and the band densities were quantified by Image J software. Data presented as mean ± SE (n = 5 to 6 for panels B and D) and the image (A) was a representative of 4 independent experiment. Means for a given measure without sharing a common letter differ (* $P <$ 0.05 vs. CTRL).

showed an increased trend in plasma glucose as compared with the control (Fig. 2.3B, right panel). Most importantly, both WT and OE pancreas exhibited a significant increase of REG2 level after DQ or STZ injection (Fig. 2.3 C, D). Collectively, these results demonstrated that REG2 protein expression was induced by oxidative stress.

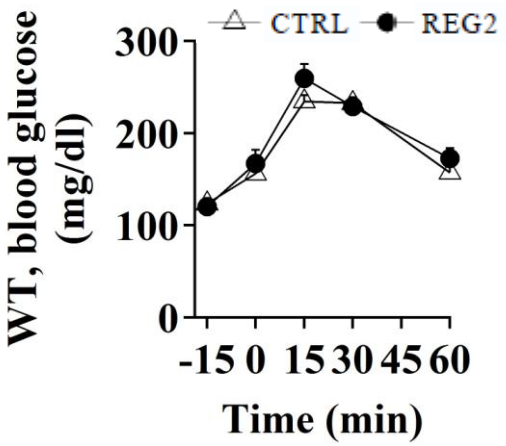
2.4.4 Acute Injection of REG2 Decreased Glucose Tolerance and GSIS in OE Mice

A low level of REG2 in OE islets also directed us to investigate the role of REG2 as an important mediator of metabolic disorders in OE mice. To examine the effect of exogenous REG2 on glucose homeostasis, we expressed and purified the mouse REG2 protein from *P. pastoris* GS200 and confirmed the protein expression (Fig. 2.4A). In order to study the role of REG2 in the OE T2DM-like phenotypes, we administered the exogenous REG2 protein to WT and OE mice and examined the blood glucose and insulin changes. To determine the acute effect of REG2 on glucose tolerance and GSIS, WT and OE mice were injected with 2 μg/g REG2, and after 15 minutes, they were challenged with 1 g/kg glucose. Blood glucose and plasma insulin level were measured at different time points. In comparison to the PBS-injected OE mice, the REG2-injected OE mice showed glucose intolerance (Fig. 2.4C) and decreased GSIS (Fig. 2.4D). However, WT mice did not show a significant response to REG2 injection (Fig. 2.4B). To summarize, these *in vivo* studies showed the acute inhibitory role of exogenous REG2 administration on GSIS in OE mice.

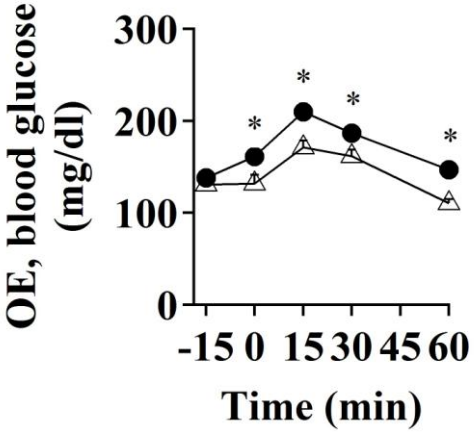
A.


REG2

B.


CTRL
REG2
WT, blood glucose (mg/dl)
300
200
100
0
-15 0 15 30 45 60
Time (min)

C.


OE, blood glucose (mg/dl)
300
200
100
0
*
*
*
*
-15 0 15 30 45 60
Time (min)

D.

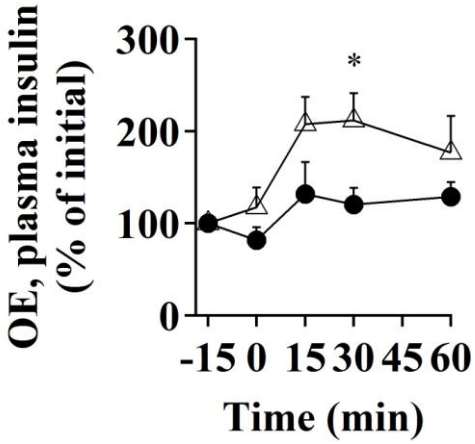
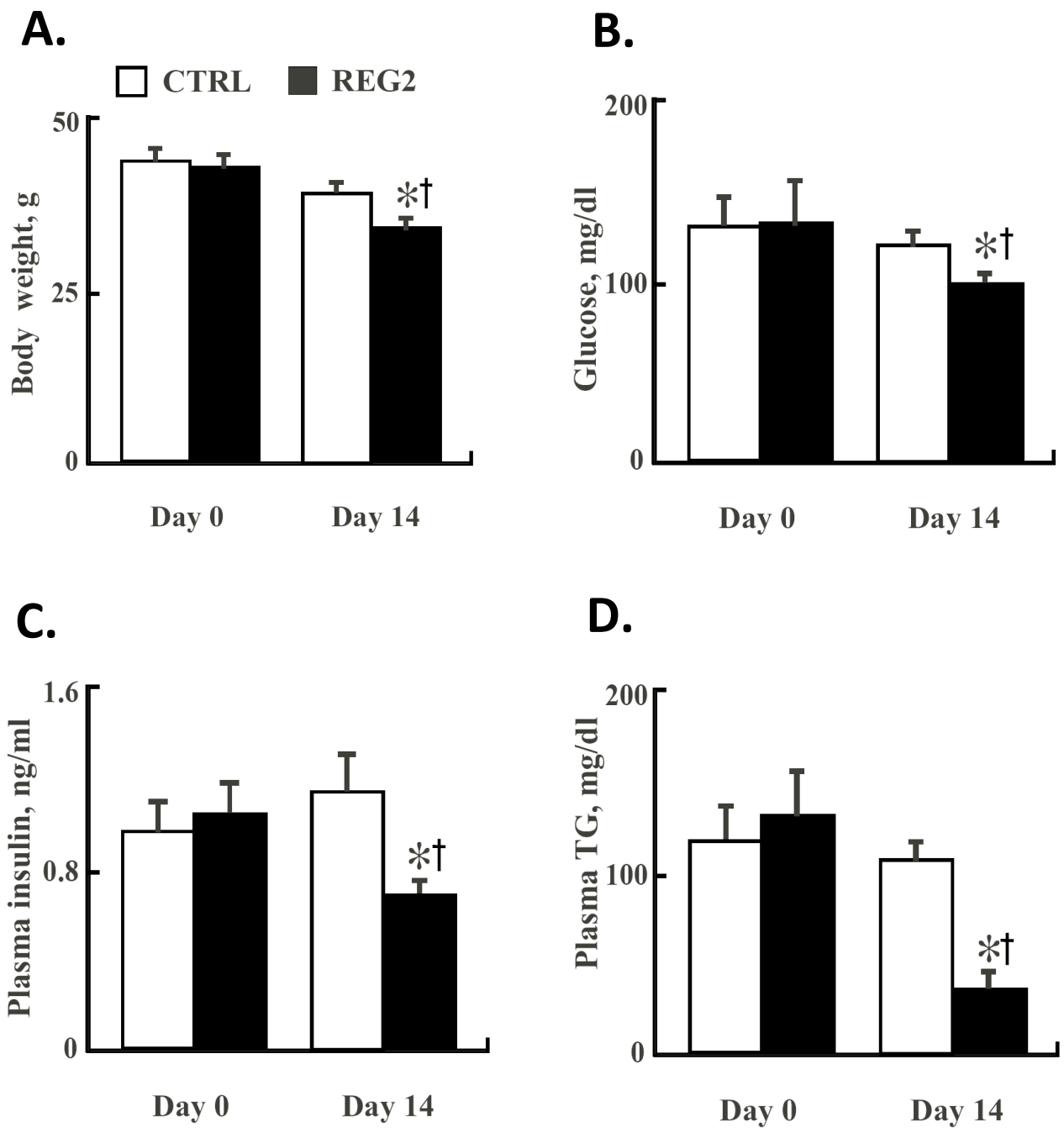

OE, plasma insulin (% of initial)
300
200
100
0
*
-15 0 15 30 45 60
Time (min)

FIGURE 2.4 Effects of short-term exogenous REG2 pre-injection on GSIS *in vivo*.

(A) The recombinant mouse REG2 protein was expressed in *P. pastoris* GS200, purified by gel filtration and ion exchange chromatography, and then redissolved in PBS buffer. The expression of REG2 protein was confirmed by immunoblotting analysis. (B, C) Effect of pre-injected REG2 on GTT results: 2 μg REG2 protein/g body weight was i.p. injected into WT and OE mice, 10ul PBS/g body weight injection group served as control. After 15 minutes, 1 g/kg glucose was i.p. injected into the mice. Blood glucose was measured at different time points. (D) Effect of pre-injected REG2 on GSIS in OE mice: 2 μg REG2 protein/g body weight was i.p. injected into OE mice, 10 ul PBS/g body weight injection group served as control. After 15 minutes, 1 g/kg glucose was i.p. injected into the mice. Plasma insulin was measured at different time points. Data presented as mean ± SE (n = 5 to 6 for panels B, C and D). Means for a given time-point without sharing a common letter differ (* $P < 0.05$ vs. CTRL).

2.4.5 Long-term Administration of REG2 Protein Improved Hyperinsulinemia, Hyperglycemia and Hypertriglyceridemia in OE Mice

To determine whether or not the REG2 protein is involved in the development of the T2DM-like phenotypes in OE mice, 1-year-old OE male mice were i.p. injected with either the recombinant REG2 protein (2 μg/g) or PBS (10 μl/g) for 14 consecutive days. Interestingly, after 14 days of daily exogenous REG2 administration, the fasting blood glucose, plasma insulin levels, body weight and plasma triglyceride (TG) were decreased compared with their day 0 baseline ($P < 0.05$) (Fig. 2.5A, B, C and D). These parameters were also significantly lower than their PBS control group ($P < 0.05$) (Fig. 2. 5A, B, C and D). The REG2 treatment group that was subjected to a glucose tolerance test exhibited glucose intolerance (Fig. 2.5E, F) and decreased GSIS (Fig. 2.5G, H), as compared with the control group subjected to the same test. However, the REG2 treatment group and PBS control group did not show any differences in insulin tolerance (Fig. 2.5I, J), indicating that the glucose intolerance by REG2 administration was caused by decreased insulin secretion.


A.
CTRL
REG2
Body weight, g
50
25
0
*†
Day 0
Day 14
B.
Glucose, mg/dl
200
100
0
*†
Day 0
Day 14
C.
Plasma insulin, ng/ml
1.6
0.8
0
*†
Day 0
Day 14
D.
Plasma TG, mg/dl
200
100
0
*†
Day 0
Day 14

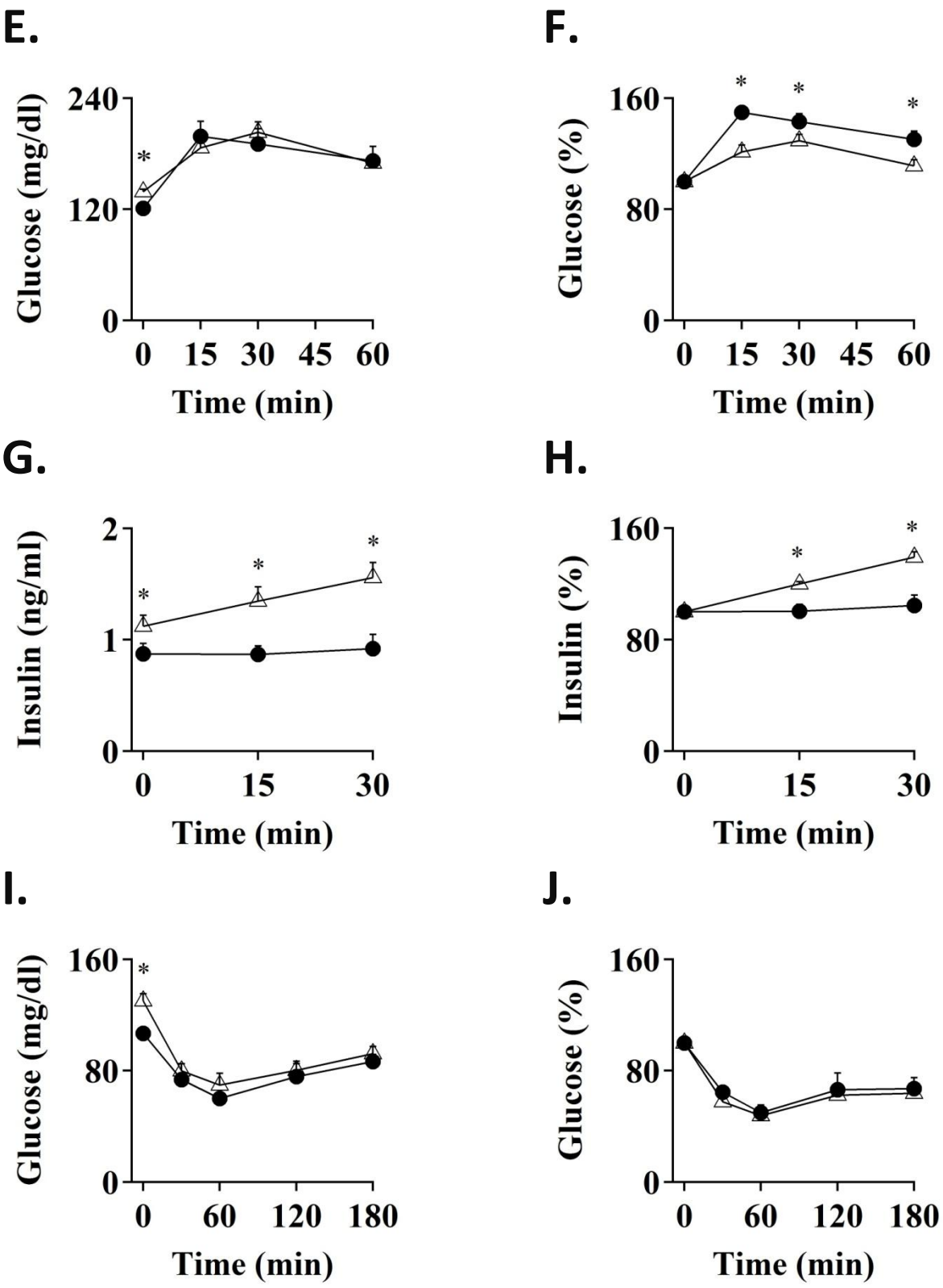

E.
Glucose (mg/dl)
240
120
0
0 15 30 45 60
Time (min)
F.
Glucose (%)
160
80
0
0 15 30 45 60
Time (min)
G.
Insulin (ng/ml)
2
1
0
0 15 30
Time (min)
H.
Insulin (%)
160
80
0
0 15 30
Time (min)
I.
Glucose (mg/dl)
160
80
0
0 60 120 180
Time (min)
J.
Glucose (%)
160
80
0
0 60 120 180
Time (min)

FIGURE 2.5 Effects of long-term daily REG2 injection on T2DM-like phenotypes in 1-year-old OE mice.

Effects of two-week daily REG2 protein i.p. injection (2 μg REG2 protein/g body weight) on (A) fasting body weight, (B) blood glucose, (C) plasma insulin, (D) plasma TG, (E, F) body glucose tolerance absolute values (left) and % of initial (right) (1 g glucose/kg), (G, H) GSIS absolute values (left) and % of initial (right) (1 g glucose/kg), (I, J) body insulin tolerance absolute values (left) and % of initial (right) (0.5 insulin U/kg), Data presented as mean ± SE (n = 5 to 6). Means for a given measure without sharing a common letter differ (* $P < 0.05$ vs. CTRL, † $P < 0.05$ vs. initial).

2.5 Discussion

Here we gained new insights into the biochemical mechanisms of GPX1 overexpression-dependent decrease of REG2 protein level in islets. We discovered the novel function of REG2 in alleviating hyperinsulinemia, hyperglycemia, and hypertriglyceridemia in old OE mice, the first study that links the antioxidant system to islet regenerating proteins.

The main function of GPX1 is to reduce intracellular H_2O_2. GSH is the substrate of GPX1 that participates directly in the neutralization of free radicals and reactive oxygen compounds. Reg2 was reported to be related to acute pancreatitis (Huszarik et al., 2010; Lu et al., 2006; Zhong et al., 2007), and its promoter activity can also be significantly increased by glucocorticoids and IL-6 treatment (Luo et al., 2013). Strikingly, mRNA level of *Reg2* was found to be significantly depleted in the islets of OE mice as compared to WT mice (Fig. 1.5A, B). In addition, REG2 expression was found to be inversely related to GPX1 expression in high-fat diet fed mice (Qiu et al., 2005). Based on the inverse correlation between GPX1 and REG2 and their connection to oxidative stress, we hypothesized that GPX1 down-regulated REG2 through ROS degradation. To test the hypothesis, we first studied the tissue distribution of REG2 protein in WT mice. We observed the highest protein expression of REG2 in the pancreas, 160 fold higher than in the liver and intestine, the organs that showed the second highest REG2 expression (Fig. 2.1A, B). This implied that the pancreas might be the main organ where REG2 expressed and functioned. To investigate further the distribution of REG2 in the pancreas, we used the immunohistochemistry approach to stain the WT pancreas section with REG2 antibody. Interestingly, we found that REG2 protein was mainly expressed in the islets rather than in the exocrine pancreatic tissues (Fig. 2.1C). We then confirmed the diminished REG2 protein expression in OE islets by using immunoblotting and immunoprecipitation methods (Fig. 2.1D,

E). These findings led us to use pancreatic islets as the *in vitro* model to study the regulation and function of REG2 protein. By treating islets with various chemicals that alter intracellular ROS levels, we demonstrated that REG2 protein expression was decreased by antioxidant treatments and increased by H_2O_2 treatment, relative to their respective controls (Fig. 2.2A, B, C and D). We then injected mice with oxidative stress inducers to study the change of REG2 protein *in vivo*. 48 hours after STZ or DQ administration, we observed the up-regulated REG2 expression in the pancreas as compared to the control (Fig. 2.3C, D). This result is also supported by a study in which STZ injections significantly up-regulated REG2 in the pancreas (Lu et al., 2006). Therefore, the above REG2 expression profile studies demonstrated that REG2 protein in islets was induced by oxidative stress, and that Se-dependent GPX1 inhibited REG2 expression by modulating intracellular ROS level.

OE mice serve as a REG2-functional knockout model since REG2 is depleted in the OE islets, so it is interesting to investigate whether or not the decreased REG2 contributes to the metabolic disorders in OE mice. To address this question, we made recombinant REG2 protein and performed short-term and long-term injection studies. Firstly, we administrated the purified exogenous REG2 protein into WT and OE mice, and after 15 minutes, we challenged the mice with 1 g per kg body weight glucose. The OE mice showed glucose intolerance and decreased GSIS after REG2 injection, while WT mice did not exhibit any significant differences compared with their own PBS-injected group (Fig. 2.4B, C and D). This study presented the acute inhibitory role of REG2 on GSIS in OE mice. In light of the finding, we further studied the chronic role of the exogenous REG2 administration on the metabolic disorders in this mouse model. We injected the 1-year-old OE mice daily with 2 μg/g recombinant REG2 protein and

monitored the change of metabolic parameters after two weeks. Exogenous REG2 administration alleviated most of the metabolic disorders in OE mice except for the insulin resistance. In comparison with the PBS-injected group and their own initial baseline, the REG2-injected group showed a significant decrease in body weight, plasma glucose, plasma insulin, and plasma TG (Fig. 2.5A, B, C and D). GSIS and glucose tolerance were also decreased in the REG2-injected group as compared to the PBS-injected group (Fig. 2.5 E, F, G and H). However, we did not observe any change in insulin tolerance after REG2 injection (Fig. 2.5I, J).

These findings, coupled with the fact that REG2 was identified as a down-stream signaling partner of GPX1, as well as the novel function of REG2 in rescuing the metabolic syndrome in OE mice, strongly suggest that REG2 plays an important role in the development of T2DM-like phenotypes in OE mice. In addition, it provides new insights into a therapeutic strategy for T2DM, in particular the prediabetic state in human.

CHAPTER 3

Novel Molecular Mechanisms of REG2 in Regulating Glucose-Stimulated Insulin Secretion

3.1 Abstract

In Chapter 2, we found that REG2 improved hyperinsulinemia, hyperglycemia, and hypertriglyceridemia in OE mice. To further confirm the role of REG2 in insulin secretion and to investigate the underlying mechanisms, we examined the regulation of insulin secretion by native and mutant forms of REG2 protein *in vitro*. We also set out to indentify the unknown REG2 binding partners by using protein-protein interaction approaches. We found that REG2 treatment decreased insulin secretion stimulated by glucose ($P < 0.05$), but did not change the insulin secretion stimulated by either potassium chloride (KCl) or arginine in OE islets. In addition, REG2 mutant 1 (M1) with mutations in C-type lectin domain failed to suppress GSIS, while REG2 mutant 2 (M2) with mutations in six acidic residues inhibited GSIS ($P < 0.05$) in OE islets. To determine the REG2 downstream signaling, we found that REG2 treatment decreased the expression of genes involved in glucose metabolism or Ca^{2+} homeostasis, but not the genes related to insulin synthesis. REG2 treatment also decreased GLUT2 protein expression, GK protein activity, and islet mitochondria membrane potential, and also increased levels of UCP2 protein level ($P < 0.05$). In search of the REG2 binding partners, we studied the interaction between REG2 with four membrane proteins and eight cytosolic proteins. However, we have not yet identified the direct binding proteins of REG2. In conclusion, we have found REG2 inhibition of insulin secretion in OE islets to be a glucose-dependent process, and this work suggested that REG2 C-type lectin domain was involved as well. Further investigation is needed to identify the REG2 binding proteins.

3.2 Introduction

Glucose is an important secretagogue for insulin secretion. Glucose usage in β cells is mainly determined by glycolysis and cellular level of GK and glucose (Matschinsky, 1996), as glycogen synthesis and pentose-phosphate shunt is very limited in a β cell (Schuit et al., 1997). The key regulators of GSIS are tightly linked to glucose and mitochondrial metabolism (Malmgren et al., 2009). When the level of blood glucose rises, glucose is rapidly transported into the cell by GLUT2. The intracellular glucose is further phosphorylated by GK, which initiates glycolysis and oxidative phosphorylation (German, 1993; Henquin, 2000). This results in an increased ATP/ADP ratio (Henquin, 2000), closure of ATP-sensitive K^+ channels (Ashcroft et al., 1984; Cook and Hales, 1984), opening of the voltage-sensitive Ca^{2+} channels, and an influx of Ca^{2+} that leads to insulin exocytosis (Kelly et al., 1991). In mitochondria, a proton circuit across the mitochondrial inner membrane drives oxidative phosphorylation, coupling substrate oxidation and ADP phosphorylation, while UCP2, one of the members of the larger family of mitochondrial anion carrier proteins, contributes to proton leak in the mitochondria inner membrane, decreasing ATP production and attenuating insulin secretion in pancreatic β cells (Krauss et al., 2002; Lowell and Shulman, 2005).

KCl stimulates insulin secretion directly through membrane depolarization, which results in the opening of voltage-dependent Ca^{2+} channels, increasing cytosolic Ca^{2+} concentration and activation of effector systems responsible for exocytosis of insulin (Ohsugi et al., 2004). Arginine is another insulin secretagogue that is a cation at physiological pH and can also directly serve as a depolarizing agent (Sener et al., 2000; Thams and Capito, 1999). It stimulates Ca^{2+}

influx and insulin granules exocytosis independently of K_{ATP} channel activity in β cells (Sener et al., 2000).

The REG family of genes belongs to the Ca^{2+}-dependent lectin (C-type lectin) gene superfamily (Lasserre et al., 1994). They are a group of secretory proteins (Bacon et al., 2012; Carrère et al., 1999; Keel et al., 2009; Orelle et al., 1992; Zenilman et al., 2000) and were proposed to interact with its own putative receptors at the plasma membrane of the cells. To date, there has been only one Reg receptor identified, exostoses tumor-like gene 3 (EXTL3). EXTL3 was identified as the Reg1 receptor by Kobayoshi et al., who probed an expression library of rat pancreatic islets with rat Reg1 and isolated a cDNA that showed over 97% homology to the human EXTL3 (Kobayashi et al., 2000; Van Hul et al., 1998). hReg1α was later found to colocalize with EXTL3 at the plasma membrane of PC12 cells, hippocampla neurons, and hippocampic CA areas and cortex (Acquatella-Tran Van Ba et al., 2012; Marchal et al., 2012), and regulate neurite elongation through its putative membrane receptor EXTL3 (Acquatella-Tran Van Ba et al., 2012). Another Reg member, Reg3α was also found to be related to EXTL3. Human Reg3α (hReg3α) was demonstrated to accelerate translocation of the EXTL3 protein to the nuclear subcellular fraction in human PANC-1 cells (Levetan et al., 2008). hReg3α was also reported to be able to interact with EXTL3 in keratinocytes to mediate epidermal hyperproliferation (Lai et al., 2012).

This study was conducted to confirm the inhibitory role of REG2 on insulin secretion observed *in vivo* from Chapter 2, and to further investigate the underlying mechanisms. We firstly examined the regulation of insulin secretion by native and mutant forms of REG2 protein *in*

vitro. We then studied the gene and protein change of the related key regulators after REG2 treatment *in vitro*. Thirdly, we set out to identify the unknown REG2 binding partners by using AP-TAG binding assay, immunoprecipitation and mass spectrometry, as well as yeast two hybrid approaches.

3.3 Materials and Methods

Plasmid Construction, Expression and Purification of REG2 Mutant Proteins

The NOD mouse *Reg2* cDNA (NM_009043, G22 to A173) was a gracious gift of Dr. Gurr (Gurr et al., 2007). The *Reg2* gene was cloned flush with the Kex2 cleavage site of a modified alpha-factor secretion signal peptide (gamma) in the pPIC9 vector (Invitrogen, Carisbad, CA) using XhoI and EcoRI restriction sites. This allowed expression of REG2 without non-native amino acids at the N-terminus. This construct and vector pPICZalphaC were digested with BstBI and EcoRI, then the gamma-*Reg2*-containing fragment was ligated into the pPICZ vector backbone to create *Reg2*/pPICZgammaC. Reg2 mutants were created by site-directed mutagenesis of the *Reg2*/pPICZgammaC construct. Mutants were created through Touchdown PCR using mutagenic forward and reverse primers (Integrated DNA Technologies, Coralville, IA) with PrimeSTAR HS DNA polymerase (Takara, Otsu, Shiga, Japan) followed by Dpn1 digestion of parental DNA template (TABLE D.). Chemically competent *E. coli* were transformed via heat shock. Sequence verification was performed by Big Dye Terminator chemistry at the Cornell Biotechnology Resource Center. Isolated plasmid was transformed into the yeast *Pichia pastoris* GS200 using electroporation (BTX, Holliston, MA) and the mutants expressed and purified as for the WT REG2.

Static System for Insulin Secretion in Primary Islets

Primary islets were isolated from WT and OE mice by collegenase perfusion and cultured in RPMI-1640 for 5 hours at 37℃ in 5% CO_2. Islets were then transferred to Krebs buffer with 2.8 mM glucose at 37℃ water bath, treated with 1 μg/ml REG2 protein for 0.5 hour and then with 30 mM glucose/KCL for 1 hour (treatment group). Islets without REG2 and high glucose treatment served as a negative control group, and the ones without REG2 but with high glucose treatment served as a positive control group. Medium supernatant was collected before (0 min) and after (60 min) glucose/KCL treatment. Insulin concentration in the supernatant was measured by using ELISA kit (Crystal Chem, Downers Grove, IL). After the 1.5 hr treatment, islets were collected and homogenized in protein lysis buffer for further experiment analysis.

Perifusion System for Insulin Secretion in Primary Islets

Seventy freshly isolated OE islets were placed on a nylon filter in a plastic perifusion chamber (Millipore, Bedford, MA). The perifusion apparatus consisted of a water bath (37℃), a fraction collector (Bio-Rad, Hercules, CA), and a fast protein liquid chromatography (Bio-Rad, Hercules, CA), which allowed programmable rates of flow and glucose concentration in the perifusion. The perifusate was a Krebs bicarbonate buffer (pH 7.4) containing 2.2 mmol/l Ca^{2+} and 0.25% BSA equilibrated with 95% O_2/5% CO_2, and the perifusion flow rate was 1 ml/min. For GSIS experiment, OE islets were perifused with 1 μg/ml/min of REG2 or REG2 mutants or PBS for 15 minutes, followed by a ramp of 0-50 mM glucose per min. The maximal islet secretion response was tested at the end of each experiment with 30 mM KCL after washout of glucose. For KCL- or Arginine- stimulated insulin secretion experiments, OE islets were perifused with 1 μg/ml/min of REG2 for 15 minutes, followed by a ramp of 0-30 mM KCL or 0-25 mM arginine per min

respectively. Fractions were collected for insulin detection by using ELISA kit (Crystal Chem, Downers Grove, IL).

Quantitative Real Time PCR Analysis

Total RNA was extracted from the primary islets by using the Trizol reagent (Invitrogen, Carisbad, CA). The concentration and integrity of diluted samples were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and 1 μg (10 μl) of RNA was reverse transcribed into cDNA using 1 μl each of dNTP and Oligo(dT) in a 5 minute 65°C reaction using a thermocycler, followed by 4 μl 5x First Strand Buffer, 2 μl 0.1 M DTT, 1 μl RNase OUT, and 1 μl Superscript III at 50°C for 50 min and 70°C for 15 minutes. 1 μl of cDNA was loaded into each well of 384 well qPCR plates and was reacted with 3 μl ddH_2O, 5 μl SybrGreen PCR Master Mix and 0.5 μl forward and 0.5 μl reverse primers for 40 cycles with a Tm of 60°C. *β-actin* was used as a reference gene and the $2^{\wedge -ddCt}$ method was used for all analysis of the results (Schmittgen and Livak, 2008).

Immunoblotting

Islets were placed in a protein lysis buffer containing 25 mM Tris-HCl (pH 7.4), 10 mM Na_3VO_4, 100 mM NaF, 50 mM $Na_4P_2O_7$, 10 mM EGTA, 10 mM EDTA, 1% Triton X-100, 5 μg/ml aprotinin, 10 nM okadaic acid, and 2 mM phenylmethylsulfonyl fluoride (PMSF). After homogenization on ice, cell lysate was centrifuged at 4°C, 14000RPM, for 15 minutes. The supernatant was collected and assayed for protein quantification via BCA assay (Thermo Scientific, Rockford, IL). The rest supernatant was used for Immunoblotting analysis. For Immunoblotting, islet lysate supernatant was heated at 100°C heat block for 5 minutes and

resolved by an SDS-PAGE gel, transferred to a nitrocellulose membrane, and blocked in a 3% milk/TBST solution for 1 hour. The membrane was incubated with primary antibodies (diluted in TBST) at 4°C, overnight, washed in TBST (3 X 15 minutes), and incubated with secondary antibodies (also diluted in TBST) for 1 hour at room temperature. Following 3X washes in TBST, the signals on the membrane were detected using a Pierce ECL Immunoblottingting System (Thermo Scientific, Rockford, IL).

Enzyme Activity Assays

Glucokinase (GK) activity in islets and liver was measured in a coupled reaction with glucose-6-phosphate dehydrogenase (G6PDH) through monitoring nicotinamide adenine dinucleotide phosphate (NADPH) production by the increased rate of absorbance at 340 nm. NADP (Sigma-Aldrich, St. Louis, MO) was used as a coenzyme and G6PDH (Sigma-Aldrich, St. Louis, MO) was used as a coupling enzyme. The assay protocol is similar to that reported by Danial (Danial, Gramm et al. 2003).

Mitochondrial Potential Measurement

Freshly isolated OE islets were cultured in HANKS buffer containing 10mM glucose with PBS or 1 μg/ml REG2 for 0.5 hour. JC-1 mitochondria dye (Sigma-Aldrich, St. Louis, MO) was added into the medium and was allowed to stain the islet mitochondria for another 20 minutes. Staining buffer was used to wash away the extracellular dye twice. Islets were then used to make slide for fluorescence microscope imaging by using Axiovert 200 M fluorescent microscope. Mitochondrial membrane potential was compared based on the orange color change.

REG2-FITC Treatment in Islets

REG2-FITC was prepared by conjugating the purified native REG2 expressed in *P. pastoris* GS200 with fluorescein by incubation of REG2 with a ten-fold molar ratio of fluorescein isothiocyanate (FITC) isomer I (Sigma-Aldrich, St. Louis, MO) in 33 mM sodium carbonate, pH 9.0 for 2 hours at 25℃ in the dark. Unreacted FITC was removed by dialysis against PBS, pH 7.4 using a 14 kDa MWCO membrane. The labeling rate was calculated to be 1.9 mol FITC/mol REG2 by $E_{280}^{0.1\%}$ of 195 and 2.59, respectively. 20 isolated islets were incubated in 150 μl Hanks' balanced salt solution containing 10 mM glucose. Islets were treated with either 1 μg REG2-FITC or 1 μg REG2-FITC and 5 μg REG2 for 1, 3, and 15 hours. Untreated islets served as a control. Islets were collected, washed 3~6 times with PBS and put into a 96-well plate with 50 μl PBS in each well. Fluorescent microscopy was performed using an Axiovert 200 M fluorescent microscope.

Immunoprecipitation (IP) and Mass Spectrometry

Fresh islets/pancreas were collected from WT mice and homogenized in the protein lysis buffer by using a homogenizer. The cell lysate was then centrifuged at 11,000rpm for 10 minutes, supernatant was collected for the IP study. Incubate 500ug WT islet/pancreas whole cell lysate was incubated with protein G beads (Santa Cruz, Dallas, TX) with/without REG2 antibody at 4°C overnight. Protein G beads with just REG2 antibody served as negative control. The next day, the beads were washed five times using washing buffer and the proteins were eluted by using SDS-PAGE loading buffer to incubate at 100°C for 5minutes. The samples were then centrifuged, the supernatant was loaded into SDS-PAGE gels. Silver staining/Sypro Ruby

staiming were used to stain the SDA-PAGE gels. The bands were excised and sent for mass spectrometry analysis. (Cornell Proteomics Center, Ithaca, NY).

AP-TAG Binding Assay

HEK293T cells were seeded at 1e5 per/ml the day before transfection. PEI was then used to transfect pre-made AP vectors into the HEK293T cells. The cells were incubated in DMEM medium with 10% FBS, 1% PenStrep at 37°C incubator with 95% CO_2. After 3 to 4 days, conditioned media (CM) was collected for binding assay. AP activity in the CM was measured by using PNPP fast tablet (Sigma-Aldrich, St. Louis, MO): 1 tablet into 20 ml H_2O. 100 ul PNPP was added into a 96 well assay plate, together with 1-5 μl of CM, OD value was recorded at 405nM every minute. AP concentration was calculated by using the equation: AP concentration (nM) = change in OD/minute *3000/ μl added. β-TC3 cells were transfect with plasmid DNA of receptor of interest by using PEI. After 48 hours of incubation, the cells were washed with HBH twice and then incubated with CM for 2 hours at room temperature. Cells were further washed with ice cold HBH for three times and then fixed with 3.7% formaldehyde in PBS for 20 minutes. After fixing, cells were washed with HBH buffer again and the plates were put in 67^{o}C incubator for 5 hours. After deactivating the endogenous AP activity, cells were washed with AP buffer and developed with NBT (70 μl; stock 50 mg/ml in 70% DMF) and BCIP (70 μl; stock 25mg/ml in H_2O) in 10 ml AP buffer. Ligand-receptor binding will be observed in the color of purple.

Yeast Two Hybrid

Primers were designed for the four BD genes: *Reg2, Ntrl, Clect, Ctrl,* and eight AD genes: *Cars, Gsn, HnrnpU, Vcp, Pkc, Pi3k, Nckap1,* and *Sec23b*. PCR method was used to amplify the gene from mouse islets or liver cDNA. Restriction enzymes: SalI, EcoRI, NheI and BamHI and T4 ligase (Promega, Madison, WI) were used to digest and ligate the genes into pAD-GAL4-2.1 Phagemid vector or pBD-GAL4 Cam phagemid vector (Agilent Technologies, Santa Clara, CA). The pBD/pAD plasmids were then transformed into DH5α competent cells. The next day, the plasmids were extracted and purified by using Mini-prep kit (Qiagen, Valencia, CA). The pBD and pAD plamids were further tranformed into freshly prepared yeast competent cells (PJ69-4A and PJ69-4α, respectively). After mating the transformed yeast cells overnight, the mixture were plated on SC-Leu-Trp-His, SC-Leu-Trp-Ade or SC-Leu-Trp-His-Ade mutant plates for protein-protein interaction screening.

Statistical Analysis

Values were expressed as mean ± SE. Significance was defined as $P < 0.05$. The most of statistical comparisons between each group were performed by using one way ANOVA. In some experiments Dunnett's test was used. Statistics was calculated using SAS 6.11 software (SAS Institute, Cary, NC). Area-under-the-curve (AUC) for insulin secretion analysis was calculated by using Kaledagraph 3.l6 software (Synergy Software, Reading, PA).

3.4 Results

3.4.1 REG2 Inhibited GSIS in OE Islets

To confirm the inhibitory role of REG2 in GSIS *in vivo,* we used both static and perifusion systems to study the GSIS change after REG2 treatment *in vitro*. In the static study, OE islets

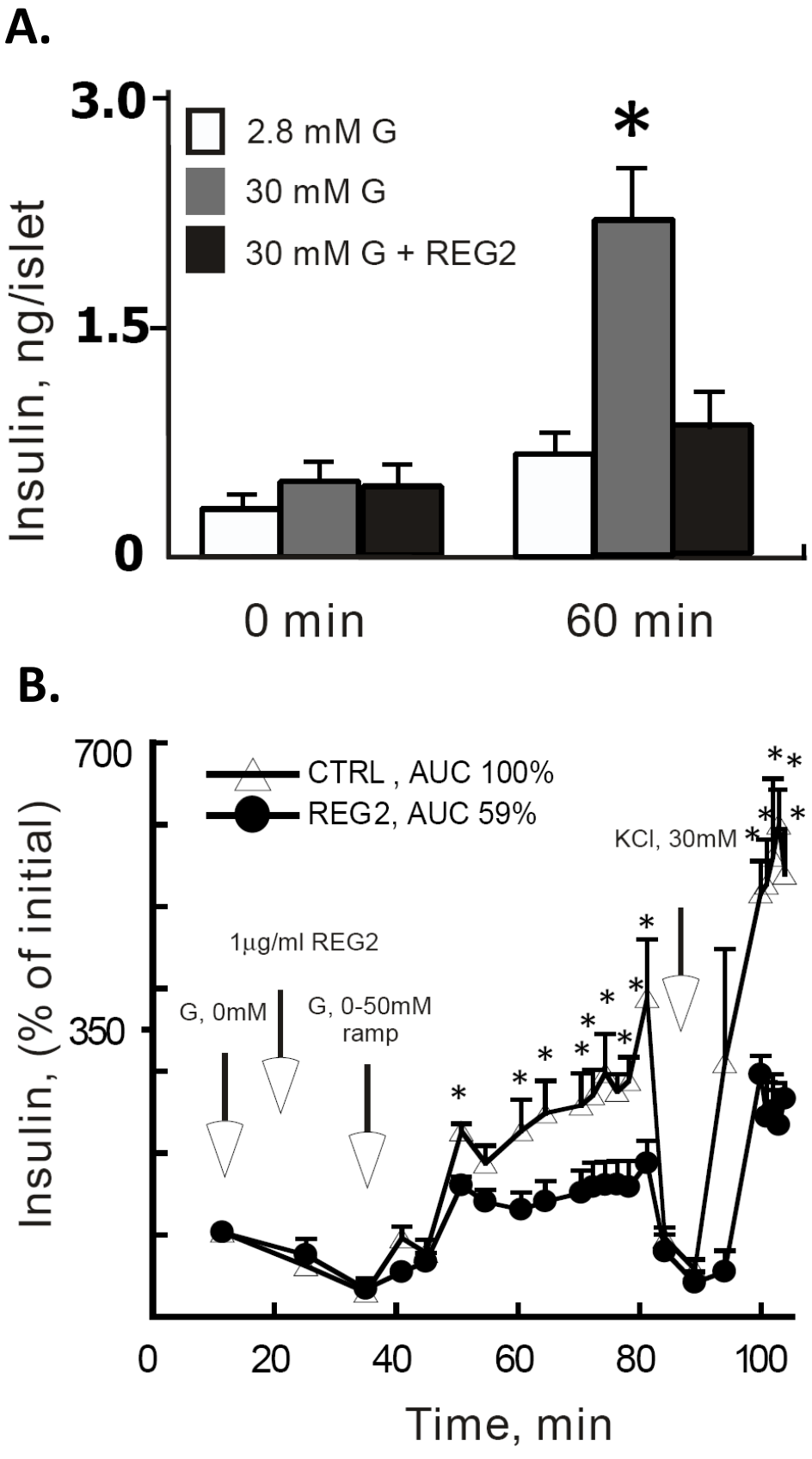
pretreated with REG2 exhibited a 59.4 % reduction *(P* < 0.05) of insulin secretion stimulated by 30 mM glucose as compared to the control group (Fig. 3.1A). In the perifusion study, OE islets were pretreated with REG2 protein or PBS for 15 minutes and followed by a 0-50 mM glucose ramp treatment. OE islets pretreated with REG2 showed suppressed insulin secretion dynamics in a glucose dose-dependent manner as compared to the PBS pretreated group (Fig. 3.1B).

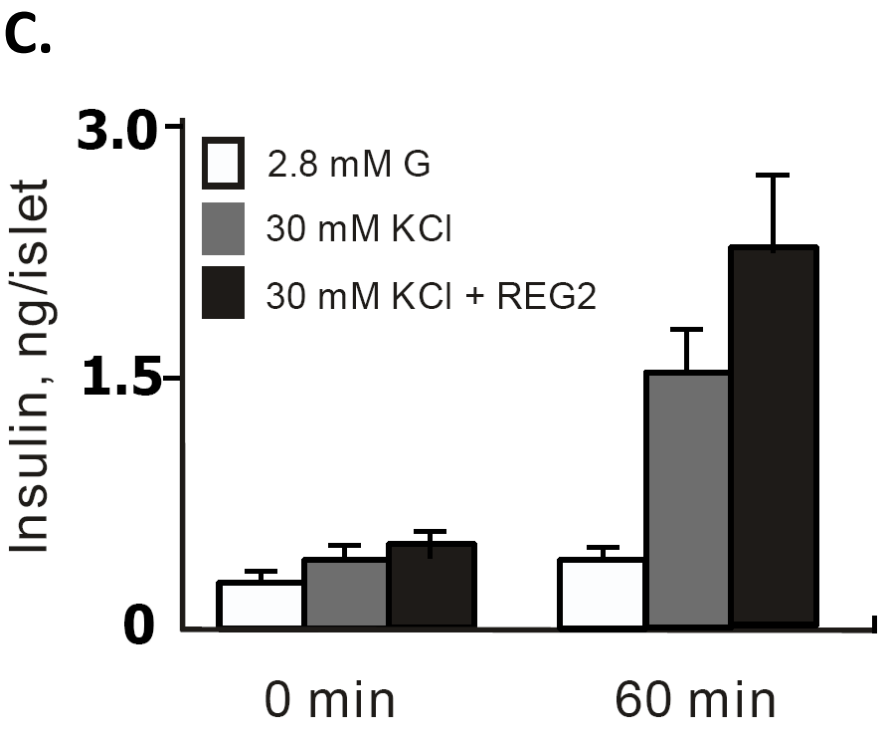
3.4.2 No Effect of REG2 Protein on KCl-Stimulated or Arginine-Stimulated Insulin Secretion

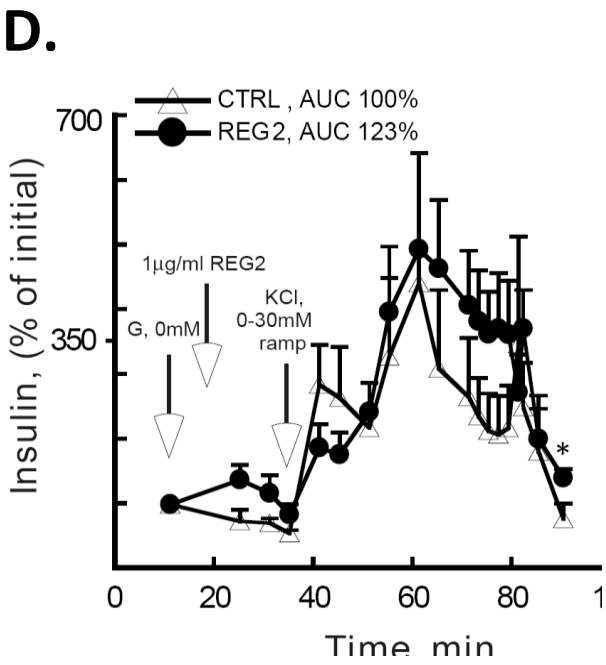
The next question was whether the inhibitory role of REG2 solely depended on glucose metabolism. To understand this, we used KCl to stimulate insulin secretion from OE islets. Interestingly, REG2 did not decrease static KCl-stimulated insulin secretion (KSIS) in OE islets with the presence of 2.8 mM glucose (Fig.3.1C). Without adding extra glucose in the medium, the dynamics of insulin secretion was not affected in the REG2-pretreated islets during 0-30 mM KCl ramp (Fig. 3.1D). Instead, KCl treatment showed some increased trend in insulin secretion in REG2-pretreated OE islets. We also tested the effect of REG2 protein on arginine-stimulated insulin secretion in OE islets. We pretreated OE islets with REG2 protein for 15 minutes, followed by 0-25 mM L-arginine ramp treatment. We found that there was no significant change of insulin secretion between REG2- and PBS-pretreated groups (Fig. 3.1E).

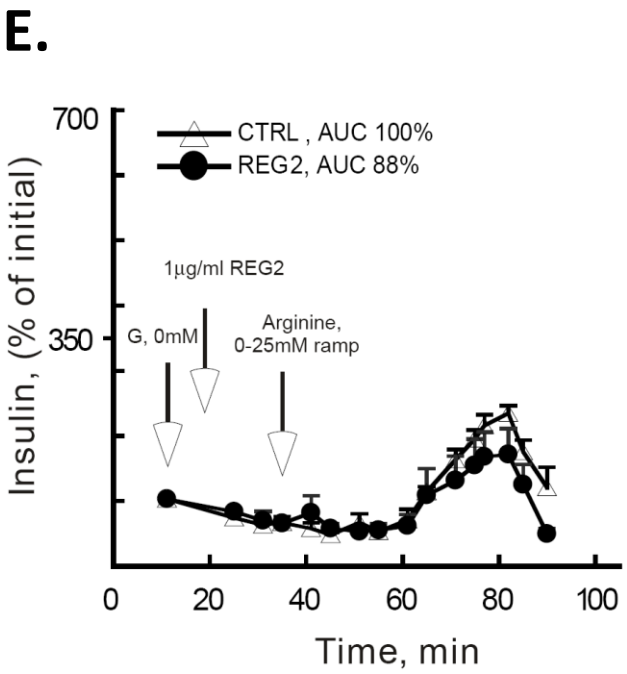
3.4.3 Different Effects of REG2 Mutants on GSIS in OE Islets

In order to understand which functional domains of REG2 might be involved in inhibition of GSIS in OE islets, we designed, expressed and purified two REG2 mutant proteins. Three amino

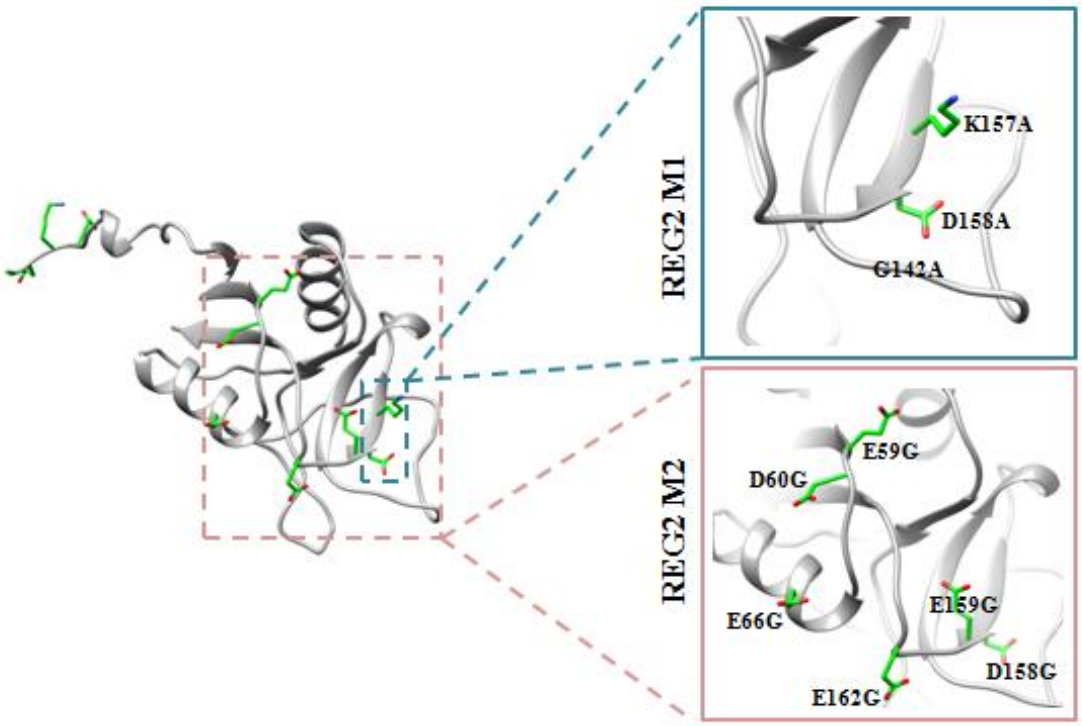

A.
3.0
1.5
0
Insulin, ng/islet
2.8 mM G
30 mM G
30 mM G + REG2
*
0 min
60 min
B.
700
350
Insulin, (% of initial)
CTRL , AUC 100%
REG2, AUC 59%
KCl, 30mM
1μg/ml REG2
G, 0mM
G, 0-50mM
ramp
0
20
40
60
80
100
Time, min


C.
3.0
1.5
0
Insulin, ng/islet
2.8 mM G
30 mM KCl
30 mM KCl + REG2
0 min
60 min

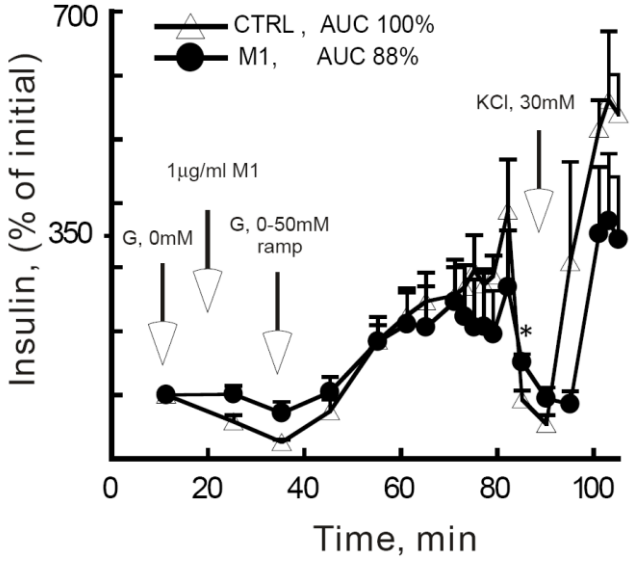

D.
CTRL , AUC 100%
REG2, AUC 123%
700
350
Insulin, (% of initial)
1μg/ml REG2
G, 0mM
KCl,
0-30mM
ramp
*
0
20
40
60
80
Time, min


E.
CTRL , AUC 100%
REG2, AUC 88%
700
350
Insulin, (% of initial)
1μg/ml REG2
G, 0mM
Arginine,
0-25mM ramp
0
20
40
60
80
100
Time, min

F.


REG2 M1
K157A
D158A
G142A
REG2 M2
E59G
D60G
E66G
E159G
D158G
E162G

G.


CTRL , AUC 100%
M1, AUC 88%
KCl, 30mM
1µg/ml M1
G, 0mM
G, 0-50mM ramp
Insulin, (% of initial)
700
350
0
20
40
60
80
100
Time, min

H.

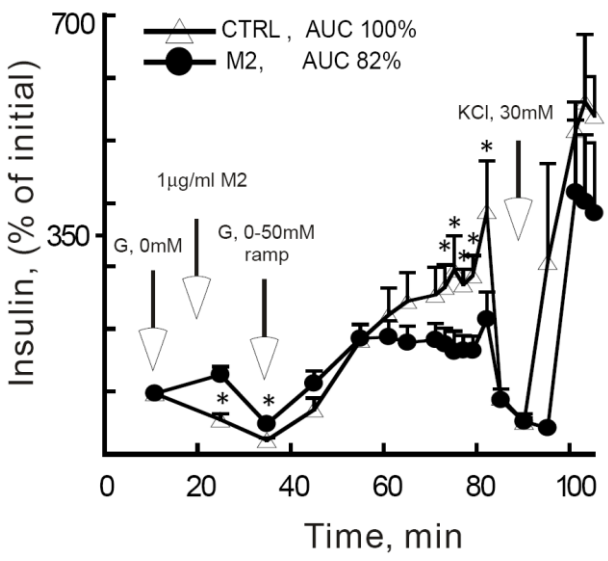

CTRL , AUC 100%
M2, AUC 82%
KCl, 30mM
1µg/ml M2
G, 0mM
G, 0-50mM ramp
Insulin, (% of initial)
700
350
0
20
40
60
80
100
Time, min

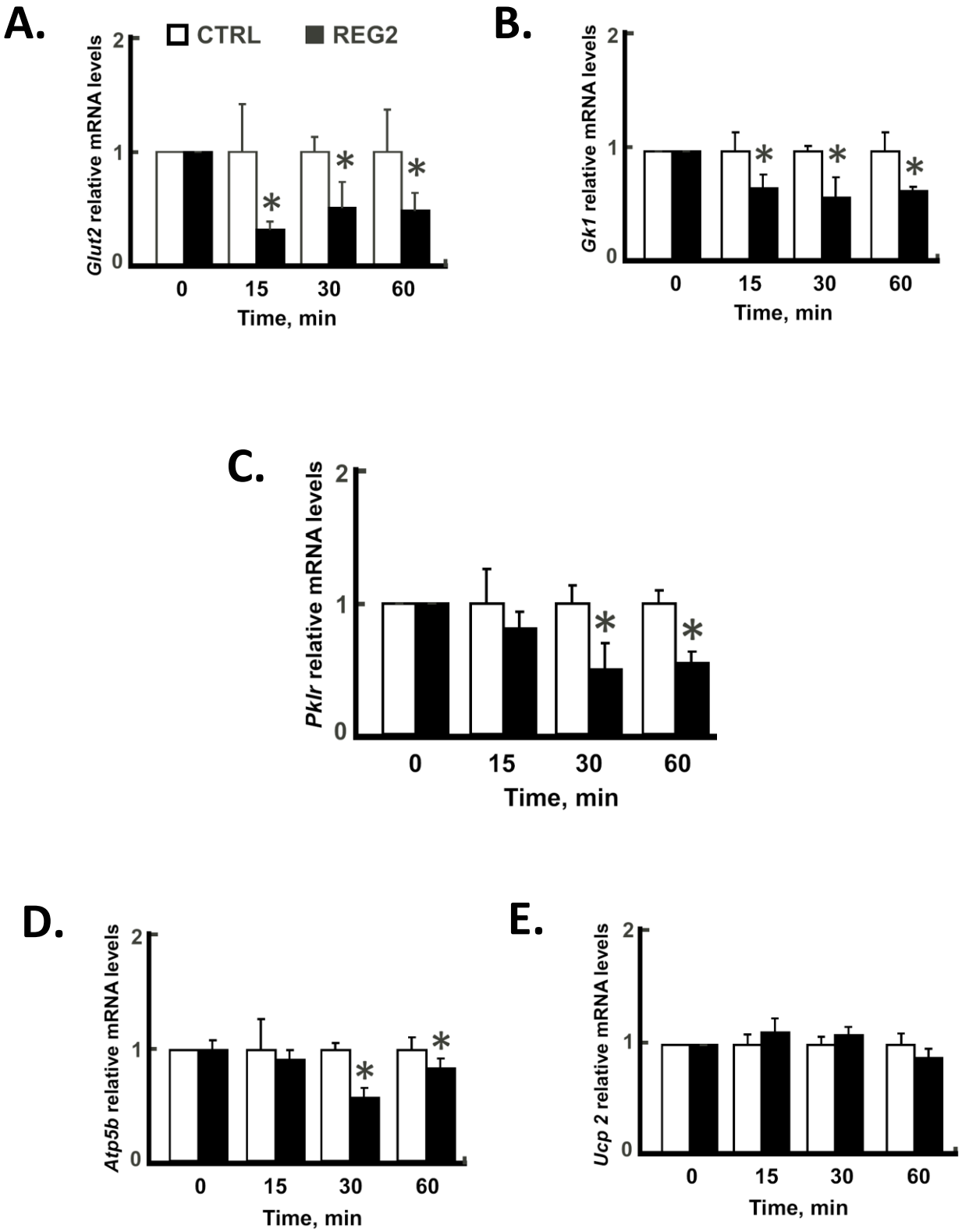
FIGURE 3.1 Effects of REG2 protein treatment on insulin secretion *in vitro*.

Effects of REG2 pretreatment on GSIS in OE islets using (A) the static incubation and (B) perifusion system. Effects of REG2 pretreatment on KCl-stimulated insulin secretion in OE islets using (C) the static incubation and (D) perifusion system. (E) Effects of REG2 pretreatment on arginine-stimulated insulin secretion in OE islets using the perifusion system. (F) Predicted three-dimensional protein structures for REG2 mutant 1 (M1) and REG2 mutant 2 (M2). Effects of (G) M1 and (H) M2 on GSIS in OE islets by using perifusion system. Data presented as mean ± SE (n = 4-5). Means for a given measure at a given time-point without sharing a common letter differ ($P < 0.05$). Area under the curve (AUC) was analyzed by using Kaledagraph software.

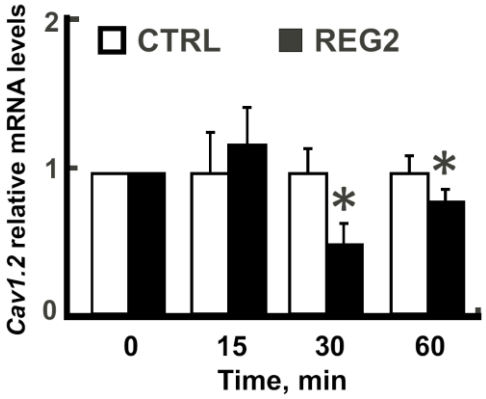
acid residues in the C-type lectin domain of REG2 (Walker et al., 2004), were mutated to alanines (G142A, K157A, and D158A) (Fig. 3.1F). We named it REG2 M1. The acidic residues potentially involved in $CaCO_3$ crystal binding (Bertrand et al., 1996) in REG2 were mutated to glycines (E59G, D60G, E66G, D158G, E159G, and E162G) (Fig. 3.1F). We called it REG2 M2. We treated OE islets with these purified mutant proteins and examined their influence on GSIS. Interestingly, compared to PBS pretreatment, REG2 M1 pretreatment did not decrease GSIS in OE islets (Fig. 3.1G), while REG2 M2 pretreatment decreased GSIS ($P < 0.05$) in a glucose-dependent manner (Fig. 3.1H).

3.4.4 REG2 Suppressed Genes and Proteins Involved in GSIS in OE Islets

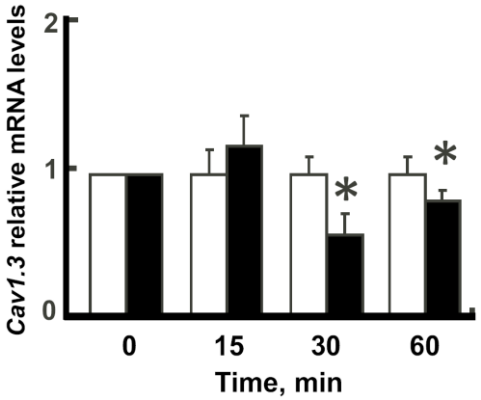
We then asked how genes changed in response to the observed inhibitory effects of REG2 treatment on GSIS. We treated OE islets with 1 μg/ml REG2/PBS and 30 mM glucose for 0, 15, 30 or 60 minutes, respectively, and examined the expression of genes related to glucose metabolism, mitochondrial metabolisms, free calcium homeostasis, as well as insulin synthesis. We observed a down-regulation of *Glut2, Gk1* after 15, 30 or 60 min ($P < 0.05$) (Fig. 3.2A, B), a decrease of pyruvate kinase, *Pklr,* and ATP-synthase subunit, *Atp5b,* after 30 or 60 min ($P < 0.05$) (Fig. 3.2C, D), but no change of *Ucp2 gene* after REG2 treatment (Fig. 3.2E). We determined a downregulation of voltage dependent L-type calcium channels, *Cav1.2* and *Cav1.3,* and protein kinase C, *Prkcb,* after 30 or 60 min of REG2 treatment ($P < 0.05$) (Fig. 3.2F, G, H). However, we were not able to detect any change in genes related to insulin synthesis, such as *Ins1*, *Foxa2*, *Beta2* and *Pdx1* (Fig. 3.2I, J, K, L). In addition to gene changes, we further tested changes in protein level of the key regulators of GSIS. We found that REG2 down-regulated GK activity ($P < 0.05$) (Fig. 3.2M) and decreased mitochondrial membrane


A.
CTRL
REG2
Glut2 relative mRNA levels
Time, min
B.
Gk1 relative mRNA levels
Time, min
C.
Pklr relative mRNA levels
Time, min
D.
Atp5b relative mRNA levels
Time, min
E.
Ucp 2 relative mRNA levels
Time, min

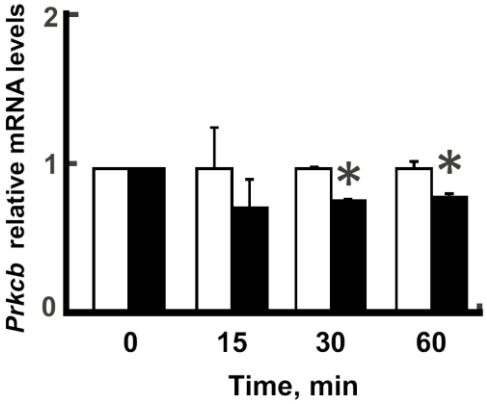
F.

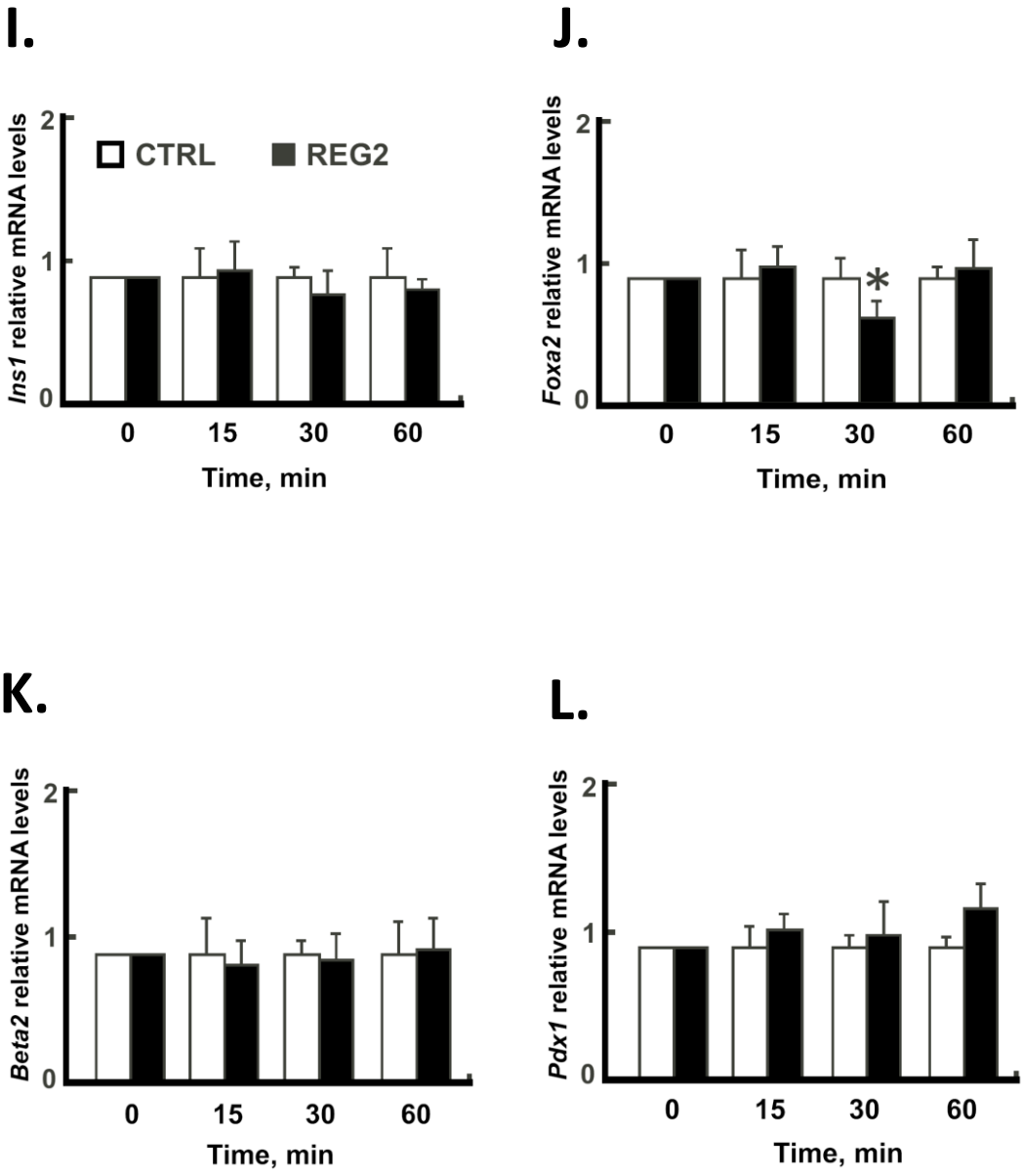

CTRL
REG2
Cav1.2 relative mRNA levels
2
1
0
*
*
0
15
30
60
Time, min

G.

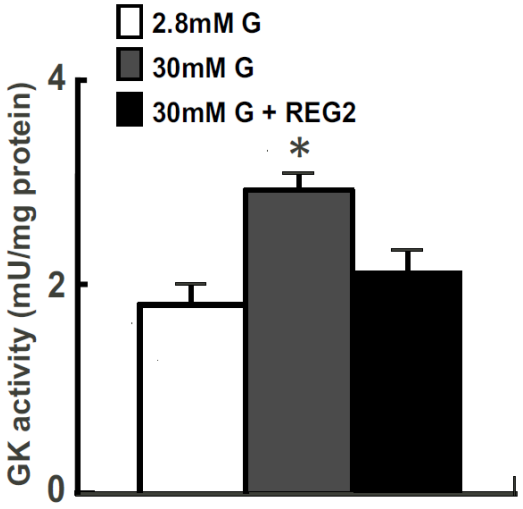

Cav1.3 relative mRNA levels
2
1
0
*
*
0
15
30
60
Time, min

H.

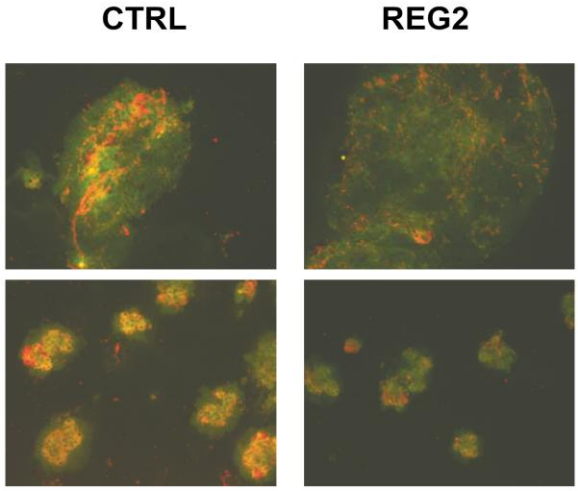

Prkcb relative mRNA levels
2
1
0
*
*
0
15
30
60
Time, min


I.
J.
K.
L.
CTRL
REG2
Ins1 relative mRNA levels
Foxa2 relative mRNA levels
Beta2 relative mRNA levels
Pdx1 relative mRNA levels
0
1
2
0
15
30
60
Time, min
*

M.


2.8mM G
30mM G
30mM G + REG2
GK activity (mU/mg protein)
4
2
0
*

N.


CTRL
REG2

O.

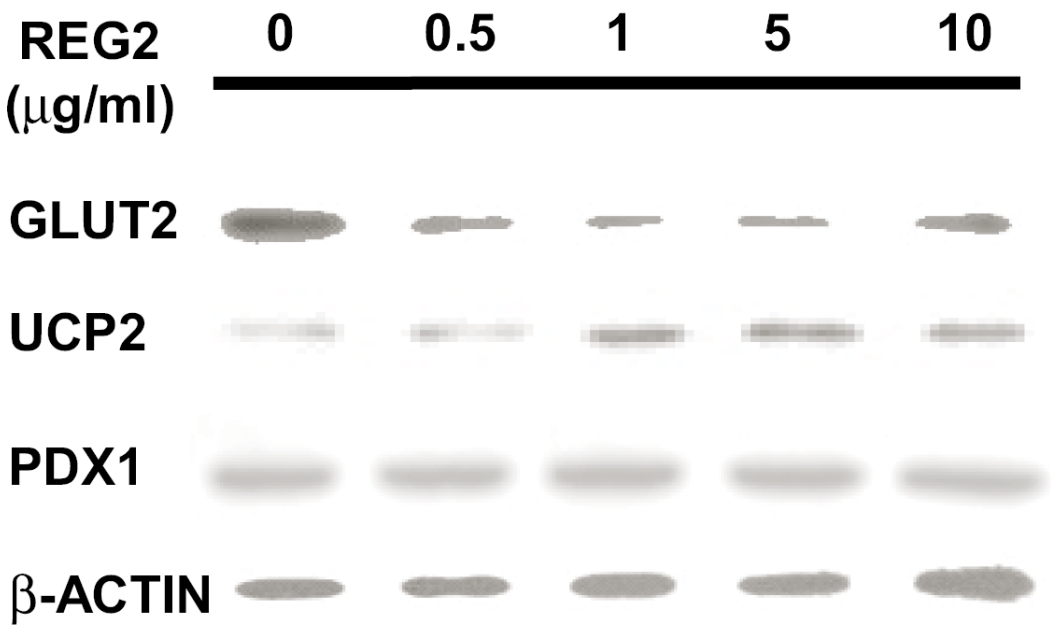

REG2 (μg/ml)
0
0.5
1
5
10
GLUT2
UCP2
PDX1
β-ACTIN

FIGURE 3.2 Effects of REG2 treatment on the expression of glucose metabolism- and Ca^{2+} homeostasis-related genes in OE islets.

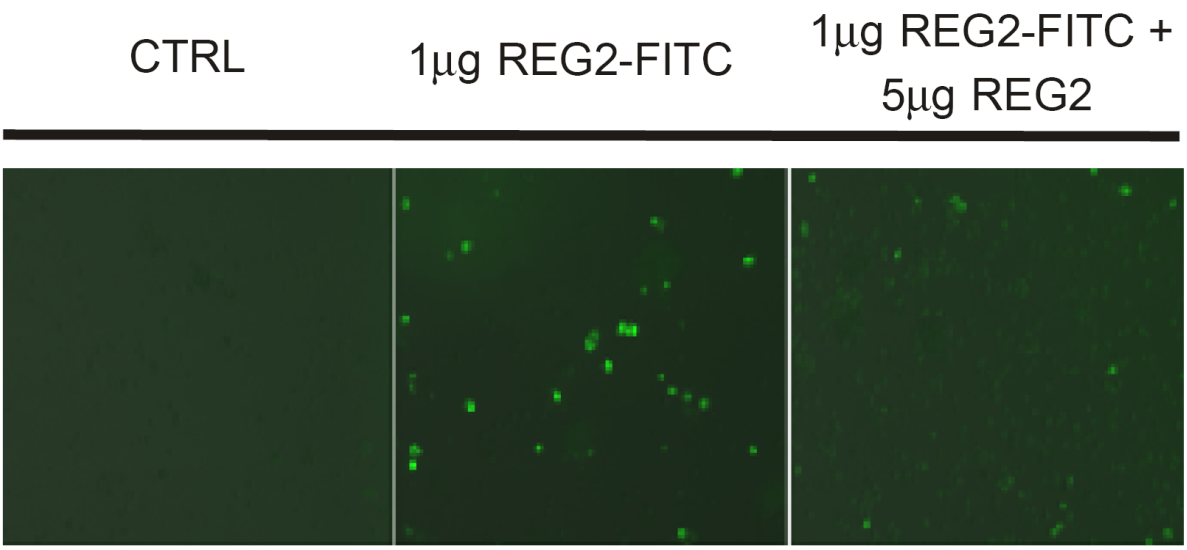
OE islets were treated with 1 μg/ml REG2 protein together with 30 mM glucose for 15, 30 and 60 minutes. Islets were collected for mRNA analysis by qPCR (A-E) Glucose metabolism-related gene expression: *Glut2*, *Gk1*, *Pklr*, *Atp5b* and *Ucp2* were analyzed. (F-H) Ca^{2+} homeostasis-related gene expression: *Cav1.2*, *Cav1.3* and *Prkcb* were analyzed. (I-L) Insulin synthesis-related gene expression: *Ins1*, *Beta2*, *Foxa2* and *Pdx1* were analyzed. (M) OE islets were treated with 1 μg/ml REG2 protein for 30 minutes following treatment with 30mM glucose for 60 minutes. Islets were collected to estimate GK protein activity. (N) OE islets were treated with 1 μg/ml REG2 protein together with 10 mM glucose for 30 minutes. Islets were further treated with JC-1 mitochondria fluorescent dye for 20 minutes and alterations of mitochondria membrane potential were detected (O) OE islets were treated with 0.5, 1, 5, 10 μg/ml REG2 protein for 30 minutes, and following treatment with 30 mM glucose for 60 minutes. Islets were collected and GLUT2, UCP2, PDX1 and β-ACTIN protein levels were analyzed by immunoblotting. Data presented as mean ± SE (n = 5) and the image (M) was a representative of 3 independent experiment. Means for a given measure without sharing a common letter differ (* $P < 0.05$ vs. CTRL).

potential (Fig. 3.2N). REG2 also dose-dependently decreased GLUT2 protein expression and increased UCP2 protein level, but not PDX1 expression (Fig. 3.2O).

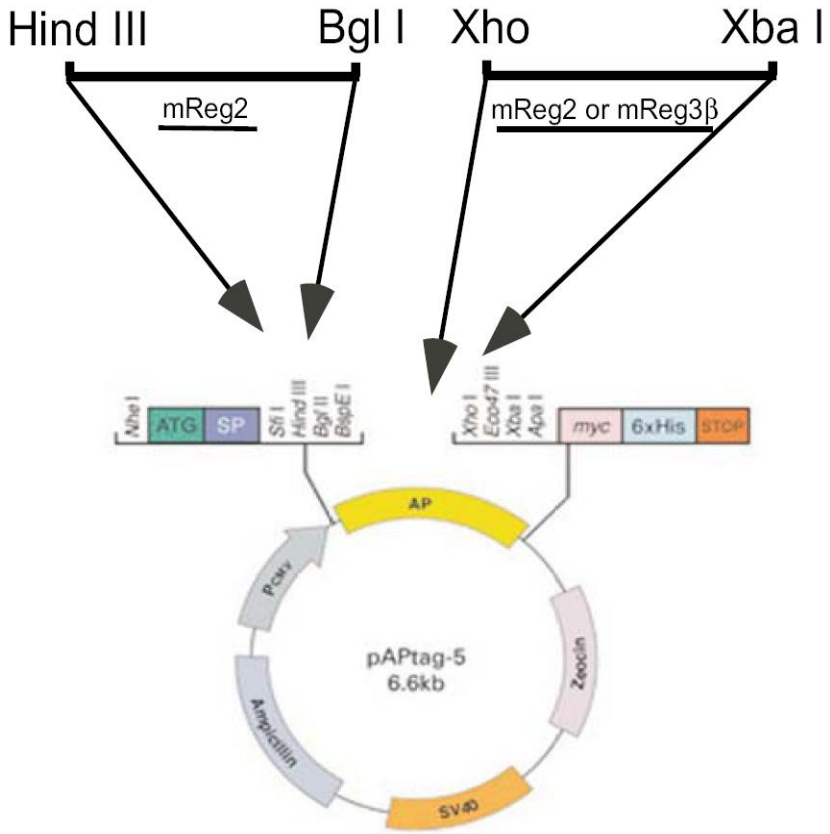
3.4.6 Identification of REG2 Interacting Proteins

Because the REG proteins are secretory proteins (Bacon et al., 2012; Carrère et al., 1999; Keel et al., 2009; Orelle et al., 1992; Zenilman et al., 2000), and some of them can interact with the membrane protein EXTL3 (Acquatella-Tran Van Ba et al., 2012; Kobayashi et al., 2000; Lai et al., 2012; Marchal et al., 2012), we investigated whether or not REG2 could bind to EXTL3 as well. To determine the general binding of REG2 on cell surface, we conjugated the recombinant REG2 protein with fluorescein isothiocyanate (FITC) and treated WT islets with the REG2-FITC protein or with a mixture of REG2-FITC and unlabeled REG2 protein. Islets without treatment served as the control. As expected, we observed a significant increase of green fluorescence in the REG2-FITC-treated islets when compared with the control (Fig. 3.3A). We also detected a reduced FITC signal when islets were treated with the mixture of FITC labeled/unlabeled REG2 (Fig. 3.3A), thus suggesting a competitive binding of REG2 to its potential membrane target. To investigate the binding of REG2 and EXTL3, we used an AP-TAG binding assay. The pAPtag-5 vector encoding mouse *Reg2* or *Reg3β* was created by inserting the gene between the restriction sites as shown (Fig. 3.3B). The constructs were transfected into HEK293T cells to make the conditioned medium. AP protein expression (Fig. 3.3C) and REG2 protein expression (Fig. 3.3D) were confirmed by using the immunoblotting assay. EXTL3 were overexpressed in β-TC3 cells, and empty pcDNA3.1 vector served as the negative control (Fig. 3.3E). After a 2-hour binding with AP conditioned mediums, AP-REG3β showed positive binding with EXTL3, as we can see the cells colored dark purple (Fig. 3.3E). Neither AP-REG2 nor REG2-AP showed any

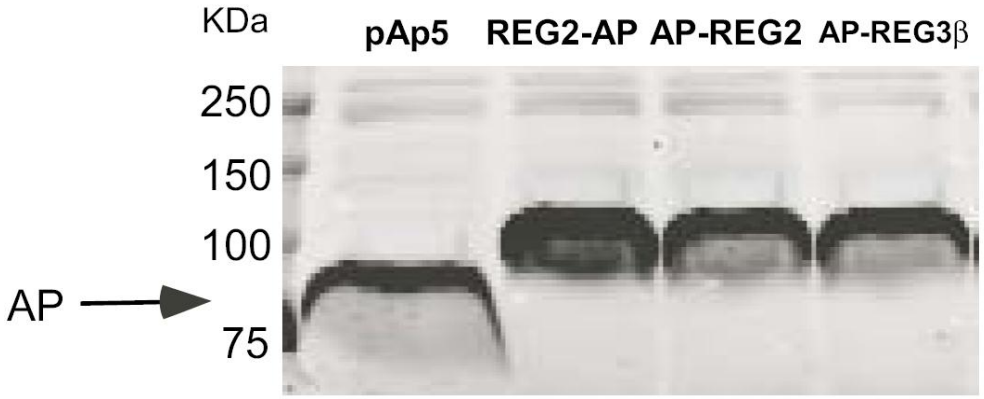
A.


CTRL
1μg REG2-FITC
1μg REG2-FITC +
5μg REG2

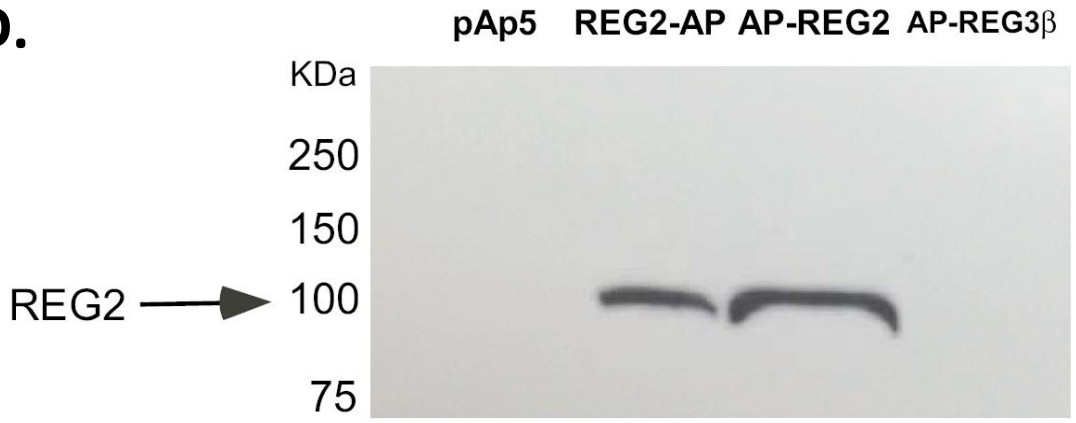
B.


Hind III
Bgl I
Xho
Xba I
mReg2
mReg2 or mReg3β
Nhe I
ATG
SP
Sfi I
Hind III
Bgl II
BlpE I
Xho I
Eco47 III
Xba I
Apa I
myc
6xHis
STOP
AP
Pcmv
pAPtag-5
6.6kb
Zeocin
Ampicillin
SV40

C.


KDa
pAp5
REG2-AP
AP-REG2
AP-REG3β
250
150
100
AP
75

D.


pAp5
REG2-AP
AP-REG2
AP-REG3β
KDa
250
150
REG2
100
75

E.

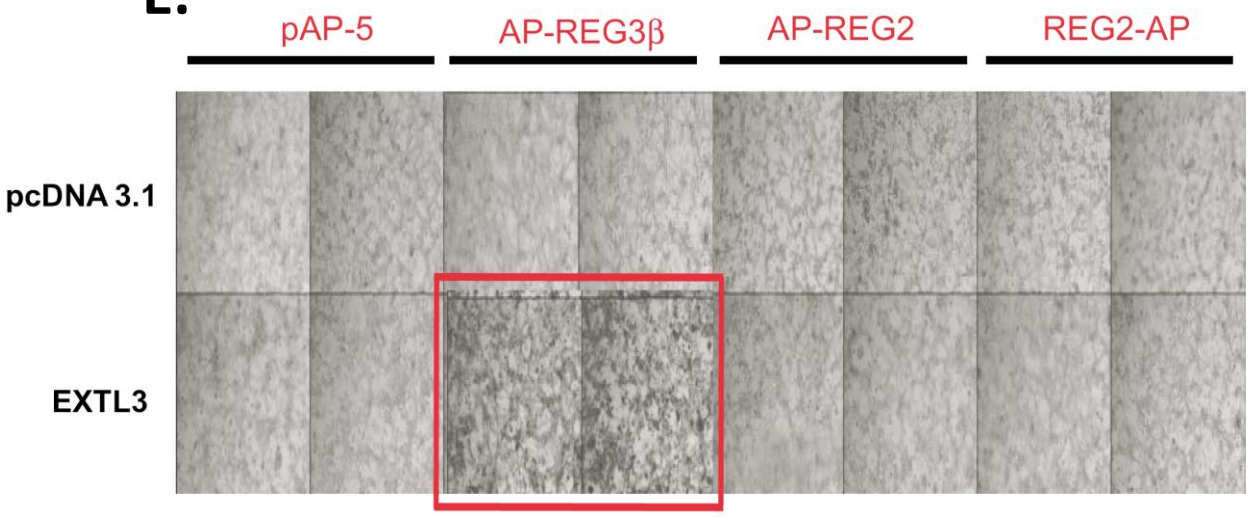

pAP-5
AP-REG3β
AP-REG2
REG2-AP
pcDNA 3.1
EXTL3

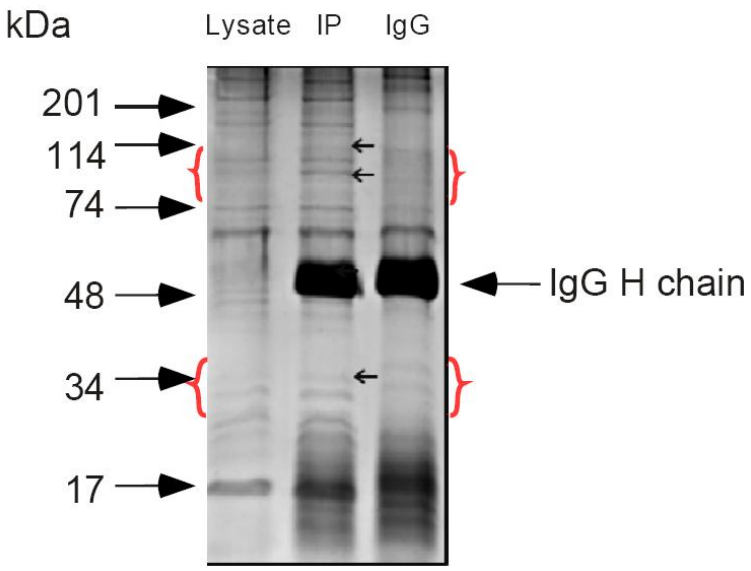
FIGURE 3.3 Identification of REG2 binding to EXTL3

(A) WT islets were treated with 1 μg REG2-FITC or 1 μg REG2-FITC + 5 μg REG2 for 15 hours. Untreated islets served as a negative control. REG2 binding on the islet surface was visualized by fluorescent microscopy. (B) Design of pAPtag-5 vector encoding mouse *Reg2* or *Reg3β* gene. The pAPtag-5 vector encoding mouse *Reg2* was created by inserting the entire coding region of the gene between XhoI and XbaI, or Hind III and Bgl II. The vector encoding mouse *Reg3β* was created by inserting the gene between XhoI and XbaI. (C) Confirmation of AP protein expression and (D) REG2 protein expression in the AP conditioned mediums by using the immunoblotting assay. (E) β-TC3 cells transfected with EXTL3 or vector control (pcDNA3.1) were incubated with conditioned medium containing 200 nM pAP5, AP-REG3β, AP-REG2 or REG2-AP protein. Binding was visualized with AP substrate BCIP/NBT. (n=4 to 5 per group).

positive binding with EXTL3, as the cells presented the same light colored background as the negative controls, and no dark purple areas are observed on these cells (Fig. 3.3E). Next, to look for proteins that preferentially interact with REG2 protein, we performed immunoprecipitation by using REG2 antibody. After pulling down endogenous REG2 and its binding partners from WT pancreas (Fig. 3.4A) or islets (Fig. 3.4B), the protein bands were excised from the gel and analyzed by mass spectrometry. We selected the candidates based on their top hits from the mass spectrometry and their involvement in actin cytoskeleton pathway or Ca^{2+} pathway, as these pathways are important for GSIS in islet β cells (Draznin, 1988; Wang and Thurmond, 2009). Among the top candidates, there are three plasma membrane proteins: epidermal growth factor receptor (EGFR), integrin beta 1 (ITGβ1), integrin alpha 5 (ITGα5), and eight cytosolic proteins: valosin-containing protein (VCP), protein kinase C (PKC), phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 isoform 2 (PI3K), NCK-associated protein 1 (NCKAP1), Cysteinyl-tRNA synthetase (CARS), Gelsolin (GSN), Heterogeneous nuclear ribonucleoprotein U (HNRNPU) and Sec23 homolog B (SEC23B). To confirm their one-to-one interaction with REG2, we used the AP-TAG binding assay to investigate the direct ligand-receptor binding of AP-REG2 or REG2-AP and the three membrane proteins on the cell surface. We also took advantage of the large-screen approach, yeast two hybrid, to confirm the interaction between REG2, REG2 N-terminal (Ntrl), REG2 C-type lectin domain (Clect), or REG2 C-terminal (Ctrl) and the eight cytosolic proteins. We have not yet been able to confirm the direct binding by using either the AP-TAG binding assay (Fig. 3.4C) or yeast-two-hybrid method (Fig. 3.4D, E). Alternative methods will be needed to confirm the direct interaction.

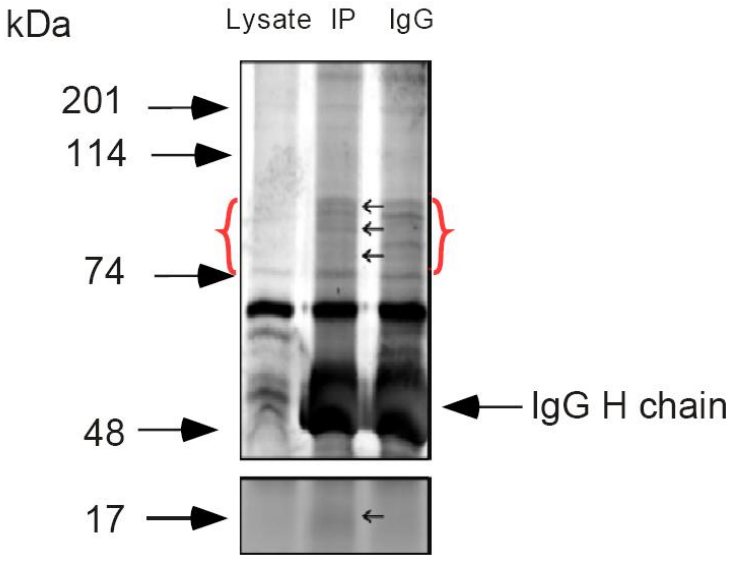
A.

Pancreas

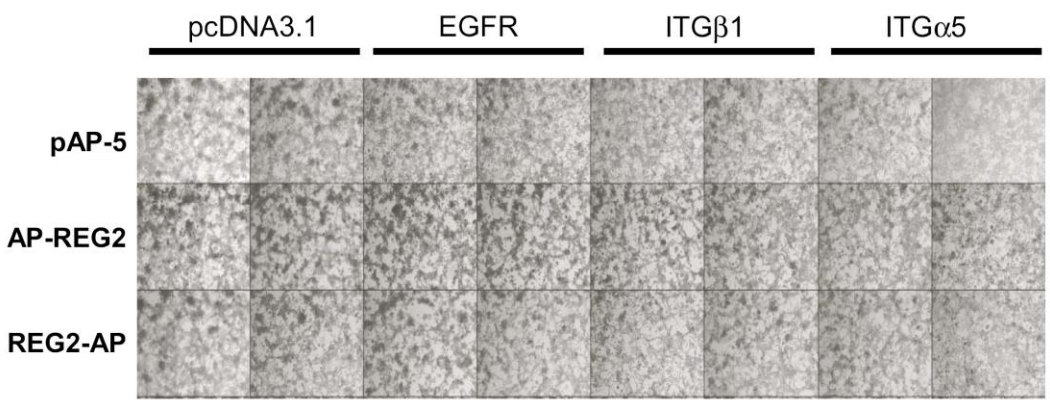


B.

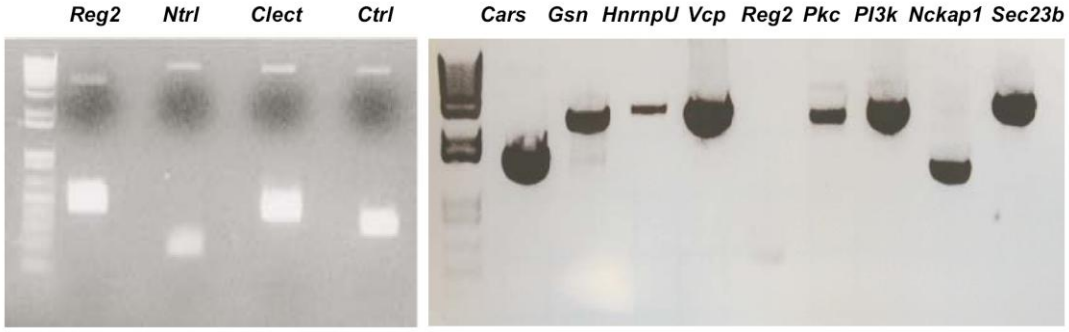
Islets



C.


pcDNA3.1
EGFR
ITGβ1
ITGα5
pAP-5
AP-REG2
REG2-AP

D.


Reg2
Ntrl
Clect
Ctrl
Cars
Gsn
HnrnpU
Vcp
Reg2
Pkc
Pl3k
Nckap1
Sec23b

E.

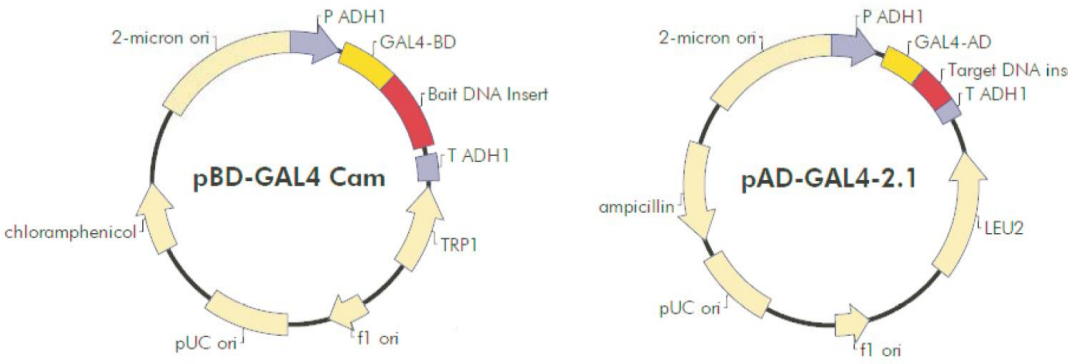

2-micron ori
P ADH1
GAL4-BD
Bait DNA Insert
T ADH1
TRP1
f1 ori
pUC ori
chloramphenicol
pBD-GAL4 Cam
2-micron ori
P ADH1
GAL4-AD
Target DNA ins
T ADH1
LEU2
f1 ori
pUC ori
ampicillin
pAD-GAL4-2.1

FIGURE 3.4 Identification of the unknown REG2 binding proteins

(A, B) Immunoprecipitation (IP) was carried out using anti-REG2 antibody mixed with protein G beads and the whole cell lysate from WT pancreas or islets. Cell lysate with protein G beads or anti-REG2 antibody with protein G beads served as negative controls. After washing, the eluted samples were loaded into SDS-PAGE gel and the gels were stained with Sypro Ruby dye. Extra bands from IP lane were cut for mass spectrometry analysis to identify potential REG2 binding proteins. (C) β-TC3 cells transfected with EGFR, ITGβ1, ITGα5 or vector control (pcDNA3.1) were incubated with conditioned medium containing 200nM pAP5, AP-REG2 or REG2-AP. Binding was visualized with AP substrate BCIP/NBT. (D) Purified PCR products for yeast two hybrid, including BD genes: *Reg2, Ntrl, Clect, Ctrl*; AD genes: *Cars, Gsn, HnrnpU, Vcp, Pkc, Pi3k, Nckap1, Sec23b*. (E) Vector design for yeast two hybrid: pBD-GAL4 Cam and pAD-GAL4-2.1.

3.5 Discussion

Despite several studies linking REG2 to diabetes, little is known about the REG2 function in insulin secretion and the underlying signaling pathway(s). Here, we report for the first time on how REG2 inhibited GSIS in the islets overexpressing GPX1 and the possible functional domains of REG2 protein that are involved in this role.

We first confirmed the inhibitory role of REG2 on GSIS in OE islets under *in vitro* conditions using both static and perifusion systems. After verifying that KCl- and arginine-induced insulin release were not affected by REG2 treatment, we focused on the glucose metabolism pathway, which might explain an underlying signaling of REG2. The first key players involved in glucose metabolism are the glucose sensor system (GLUT2 and GK) that controls glucose uptake and initiates the glycolysis pathway (Meglasson and Matschinsky, 1984; Thorens and Mueckler, 2010). Several studies have implied the regulation of REG2 on GLUT2. Liu and colleagues demonstrated that REG2 protects murine islet β-cells from STZ-induced apoptosis (Liu et al., 2010), and Xiong et al. showed the possible inhibitory effects of Reg3β on GLUT2 level to block STZ from entering islet β-cells and protecting them from STZ-induced apoptosis (Xiong et al., 2011). To understand the underlying mechanism behind how REG2 suppressed GSIS, we treated OE islets with REG2 and examined the mRNA and protein change of the key regulators. We observed time-dependent inhibition of *Glut2, Gk1, Pklr, Cav1.2, Cav1.3* and *Prkcb* gene expression ($P < 0.05$) after REG2 treatment (Fig. 3.2A, B, C, F, G, H). But we did not find any change of *Ins1* and the transcription factors involved in insulin synthesis after REG2 treatment (Fig. 3.2I, J, K, L). We further examined the protein change of the key GSIS regulators. We showed REG2 treatment decreased GK activity (Fig. 3.2M), and GLUT2 protein level (Fig.

3.2O) in OE islets in comparison to their own control group, but we did not observe any change of PDX1 protein expression (Fig. 3.2O).

Glycolysis in the β cell is tightly coupled to mitochondrial oxidative metabolism and mitochondrial membrane potential (Lowell and Shulman, 2005). One of the key regulators of GSIS is mitochondrial UCP2, which contributes to the proton leak in the mitochondria inner membrane, thus altering mitochondrial membrane potential and inversely correlated with GSIS (Krauss et al., 2002). Previously, we observed higher mitochondrial membrane potential and lower UCP2 protein level in OE islets as compared to the WT (Wang et al., 2008). To determine whether the mitochondria metabolism was affected by REG2, we measured the related gene and protein expression, as well as the mitochondria membrane potential change in REG2 pretreated OE islets. Although we didn't see any gene change of *Ucp2* (Fig. 3.2E), we found that the UCP2 protein level was significantly up-regulated (Fig. 3.2O) by 1 μg/ml REG2 treatment. UCP2 is an unstable protein with a very short half-life (Rousset et al., 2007). The protein level was decreased to 50% 25 minutes after treatment of cycloheximide *in vitro* and 60 minutes after injection of the translation inhibitor in mice (Rousset et al., 2007). Both mRNA and protein expression of UCP2 were dependent on glucose metabolism, especially GK in pancreatic islets (Dalgaard, 2012) and the expression could be also up-regulated by mitochondria O_2^- (Krauss et al., 2003). These characteristics of UCP2 indicate that REG2 might affect UCP2 protein stability in a glucose-dependent way. *Atp5b* gene expression (Fig. 3.2D) and mitochondria membrane potential were also decreased (Fig. 3.2N) by REG2 treatment, implying decreased ATP production and decreased insulin secretion.

Overall, REG2 suppressed insulin secretion in a glucose-dependent manner. The REG2 treatment of OE islets caused time-dependent suppression of genes involved in glucose and mitochondria metabolism, L-type voltage Ca^{2+} channels and *Prkcb*. Furthermore, REG2 also decreased the protein level of GLUT2, GK activity, and mitochondria membrane potential, and also increased UCP2 protein level in OE islets. However, it did not change either mRNA level of *Ins1* or mRNA and protein expression of the transcription factors that regulate insulin biosynthesis. These results implied that REG2 regulated insulin level mainly through insulin secretion by glucose stimulation rather than insulin synthesis.

We then asked which domains in the REG2 protein are responsible for the inhibitory role of GSIS. The REG family of genes belongs to the Ca^{2+}-dependent lectin (C-type lectin) gene superfamily (Lasserre et al., 1994). The CTLD in REG proteins has antibacterial function (Lehotzky et al., 2010; Mukherjee et al., 2009; Mukherjee et al., 2013). This domain is involved in carbohydrate binding (Cash et al., 2006; Christa et al., 1994; Ho et al., 2010; Lehotzky et al., 2010), through which the REG lectins can recognize their bacterial targets and mediate the function of killing and phagocytosis. Although there has not been any evidence of CTLD involvement in REG2 function, this domain might play an important role in GSIS suppression. Based on the carbohydrate- and calcium-binding sites in CTLD, G142, K157, and D158 in REG2 were predicted as the ligand-binding sites (Walker et al., 2004) (Fig. 3.5). To test whether or not the three residues are involved in REG2 function, we made REG2 M1 mutant with the three amino acid residues mutated to alanines (Fig. 3.1F). Interestingly, with the mutation in CTLD, the REG2 protein treatment of OE islets did not show significant inhibitory effects on

GSIS. This implied that mutation in the CTLD led to the loss-of-function of REG2 protein in inhibiting GSIS. In other words, CTLD might serve as the functional domain of REG2 protein.

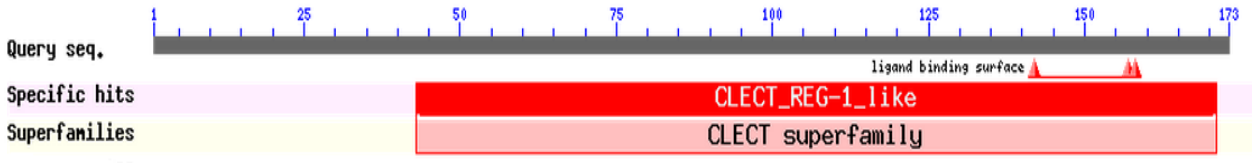


FIGURE 3.5 Predicted ligand binding surface in REG2 sequence The three amino acid residues, G142, K157, and D158, of REG2 predicted to be involved in ligand binding are shown. CD-Search was used to search the Conserved Domain Database with the REG2 amino acid sequence (Marchler-Bauer et al., 2009; Marchler-Bauer and Bryant, 2004).

Human Reg1alpha (hReg1α) was first discovered in pancreatic calcified stone (De Caro et al., 1979), and was found to inhibit $CaCO_3$ precipitation and prevent stone formation from pancreatic juice (Bernard et al., 1992; Giorgi et al., 1989; Iovanna et al., 1993; Multigner et al., 1983). The mouse Reg family protein models are shown in comparison to the hReg1α (Fig. 3.6), which has its structure determined. The acidic residues (Fig 3.6) of hReg1α were later shown to be involved in $CaCO_3$ binding (Bertrand et al., 1996) and in the control of $CaCO_3$ crystal growth (Sarles et al., 1990). Among the seven mouse Reg family proteins, the Reg1 and Reg2 models showed the greatest conservation of these acidic residues (Fig. 3.6). Based on the computational modeling prediction, as well as the involvement of calcium in insulin secretion, we hypothesized that the acidic residues (E59, D60, E66, D158, E159, E162) contributed to REG2 involvement in

the alteration of insulin secretion. To test this hypothesis, we mutated the six residues to glycine and treated OE islets with the purified recombinant REG2 M2 protein to study the change of insulin secretion. Similar to the native REG2 protein, REG2 M2 protein treatment suppressed insulin secretion in OE islets in a glucose-dependent manner. This finding shows that mutation of the acidic residues does not change the function REG2 protein on insulin secretion, indicating that these residues are not the functional domain of REG2 protein.

The Reg family is a group of secretory proteins (Bacon et al., 2012; Carrère et al., 1999; Keel et al., 2009; Orelle et al., 1992; Zenilman et al., 2000) that were found to interact with their receptor, EXTL3, at the plasma membrane of the cells (Acquatella-Tran Van Ba et al., 2012; Kobayashi et al., 2000; Lai et al., 2012; Marchal et al., 2012). However, no studies have been shown on any REG2 interacting proteins. Thus we set out to identify the unknown binding partners. Because REG2 is a secreted soluble protein, the possibility of hormone-receptor interactions was considered. We firstly used the FITC-labeled REG2 protein to study the general REG2 binding on the cell surface. As expected, we observed significant increase of green fluorescence in the REG2-FITC-treated islets when compared with the control (Fig. 3.3A), as well as the islets treated with the mixture of FITC labeled/unlabeled REG2 (Fig. 3.3A). These results suggest a competitive binding of REG2 to its potential membrane target, thus implying that REG2 interacts with certain membrane protein on the cell surface. Since rReg1, hReg1α, and hReg3α were shown to interact with EXTL3 and regulate cell function through EXTL3 (Acquatella-Tran Van Ba et al., 2012; Kobayashi et al., 2000; Lai et al., 2012; Marchal et al., 2012), it is logical to determine whether or not REG2 also binds to EXTL3.Thus far we were

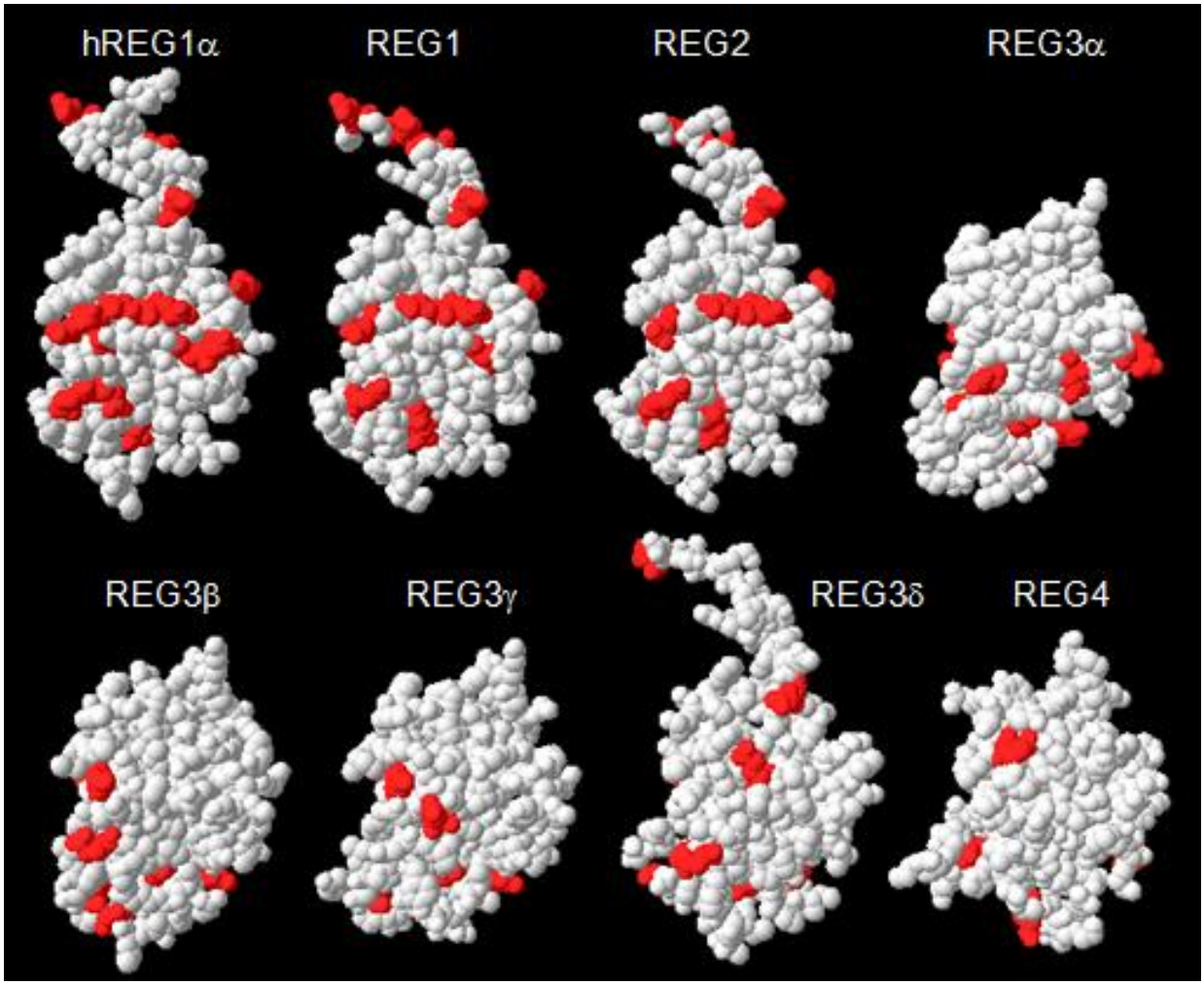


FIGURE 3.6 Predicted acidic residues of REG proteins possibly involved in $CaCO_3$ crystal binding. The mouse REG family protein models are shown in comparison to the human REG1α, which has had its structure determined (top left). The acidic residues (red) of hReg1α are involved in $CaCO_3$ binding (all other residues are white) (Bertrand et al., 1996). Among the mouse REG family proteins, the REG1 and REG2 models show the greatest conservation of these acidic residues.

unable to detect any interaction between these two proteins, as the EXTL3 overexpressing β-TC3 cells did not show positive binding to either AP-REG2 or REG2-AP (Fig. 3.3E).

To continue looking for the REG2 receptor candidates, we used REG2 antibody to immunoprecipitate the potential REG2 binding proteins, and selected three precipitated proteins: EGFR, ITGβ1 and ITGα5 based on the mass spectrometry results (Fig. 3.4A, B). EGFR is related to Reg family proteins. Although Bishnupuri group did not find a direct binding of EGFR and REG4, they found that EGFR mediated REG4-activated AKT-PI3K-AP1 signaling pathway in colon adenocarcinomas (Bishnupuri et al., 2006). There is also a strong correlation between EGFR and glucose metabolism mediated by glucose transporters. EGFR maintains basal intracellular glucose level by interacting and stabilizing sodium/glucose cotransporter 1 (SGLT1) to allow enough glucose uptake for ATP generation in cancer cells, and this is independent of its kinase activity (Weihua et al., 2008). EGFR also mediates EGF-induced alterations of glucose uptake and GLUT2 expression in hepatocytes (Lee et al., 2006) and SGLT1 expression in enterocytes (Chung et al., 1999). In addition, EGFR is transactivated by beta cellulin to enhance cell proliferation in islet β cells (Buteau et al., 2003), which demonstrated the involvement of EGFR in islet β cell function. Integrin receptors are a large family of proteins well-known for cell attachment with other cells or extracellular matrix (ECM). Integrins are heterodimeric transmembrane adhesion proteins composed of α- and β-subunits, which are critical for normal islet proliferation, differentiation and endocrine function (Bosco et al., 2000; Diaferia et al., 2013; Jiang et al., 1999; Parnaud et al., 2006; Saleem et al., 2009). Integrin β1 has been established as a positive regulator of glucose metabolism (Riopel et al., 2011) and insulin secretion (Parnaud et al., 2006; Rondas et al., 2012) in islet β cells. Integrin α5, one of the seven

α subunits associated with Integrin β1 (Krishnamurthy et al., 2008), is involved in islet β cell function as well (Wang et al., 2005). Likewise, integrins are also involved in Reg signal transduction as pancreatic REG2 protein expression was reported significantly decreased in integrin β1-deficient mice (Riopel et al., 2013) and REG3α was found to decrease the dependence of adhesion on integrins in melanoma cells (Valery et al., 2001). Because we showed that REG2 inhibited glucose metabolism and insulin secretion and immune-precipitate REG2, EGFR, Integrin β1 and integrin α5 with endogenous REG2 protein, it is logical to speculate that at least one of them is the REG2 receptor. Unfortunately, we have so far not been able to detect a physical interaction between REG2 and either one of these receptor candidates. Alternative methods might be needed to continue further investigation.

Taken together, these findings provided us with a mechanistic picture of how REG2 decreased glucose-stimulated insulin secretion in OE islets. Novel to this study is the discovery of the inhibitory role of REG2 in glucose-dependent insulin secretion OE islets *in vitro*, the downstream signaling pathway of REG2, as well as the domains that might confer the regulatory role in REG2 signaling on GSIS.

CHAPTER 4

Dietary Se Deficiency Partially Rescues Type 2 Diabetes-Like Phenotypes of Glutathione Peroxidase-1-Overexpressing Male Mice

4.1 Abstract

This study was conducted to determine if dietary Se deficiency precluded GPX1 overproduction in OE mice and rescued their T2DM-like phenotypes. A total of 20 male OE and wild-type (WT) mice were fed a Se-deficient (<0.02 mg/kg) diet or Se-supplemented (0.3 mg/kg as sodium selenite) diet from 1 to 5 months of age. Dietary Se deficiency eliminated or attenuated (P < 0.05) genotype differences in concentrations of fasting blood glucose, plasma insulin, and(or) hepatic lipids, insulin sensitivity, and glucose-stimulated insulin secretion. Dietary Se deficiency decreased (P < 0.05) OE islet mRNA levels of two key transcriptional activators (*Beta2* and *Foxa2*), and removed their differences from the WT in 6 genes (*Beta2*, *Cfos*, *Foxa2*, *Pregluc*, *Gk1* and *Sur1*) related to insulin synthesis and secretion. Depletion of Se in the OE mice also down-regulated (P < 0.05) hepatic mRNA levels of two key rate-limiting enzymes for lipogenesis (*Acc1*) and glycolysis (*Gk1*), along with activities of hepatic glucokinase and muscle phosphoenolpyruvate carboxykinase. Dietary Se deficiency affected (P < 0.05) blood glucose, plasma insulin, insulin sensitivity, and hepatic lipid profiles in the WT mice as similarly in the OE mice. In conclusion, dietary Se deficiency precluded the GPX1 overproduction in the full-fed OE mice and partially rescued their metabolic syndromes. This alleviation resulted from mitigating expression and (or) function of pro-insulin genes, lipogenesis rate-limiting enzyme genes, and key glycolysis and gluconeogenesis enzymes in islets, liver, and muscle.

4.2 Introduction

Se was considered to be anti-diabetic (Faure, 2003; Mueller and Pallauf, 2006) due to its insulin-mimetic effect (Ezaki, 1990). Indeed, Se deficiency is associated with diabetes (Akbaraly et al., 2010; Kljai and Runje, 2001; Navarro-Alarcon et al., 1999), and administration of Se to streptozotocin-induced diabetic rats restored their glycemic control (Battell et al., 1998; Chaturvedi et al., 1991). However, a number of recent human studies have shown a hyperglycemic, hyperlipidemic, and pro-diabetic effect of Se supplements (Bleys et al., 2007; Bleys et al., 2008; Stranges et al., 2010b). Our laboratory demonstrated that a prolonged feeding of high dietary Se (3 mg/kg) induced mild gestational diabetes in the first-parity pregnant rats and insulin resistance in their offspring (Zeng et al., 2012). While other groups have also shown similar (Labunskyy et al., 2011) effects of Se on body glucose and lipid metabolism, mechanisms and health impacts of such effects of Se remain unclear or controversial.

GPX1 was the first-identified selenoprotein in mammals (Rotruck et al., 1973) and has been widely considered to be a major antioxidant intracellular enzyme (Lewis et al., 2007). While GPX1 activity is extremely low in islets (Grankvist et al., 1981; Lenzen et al., 1996) and is altered in development of diabetes (Peuchant et al., 2004) and complications (Hamanishi et al., 2004; Kuzuya et al., 2008; Lewis et al., 2007; Nemoto et al., 2007), an increased activity of this enzyme in erythrocytes was actually associated with hyperinsulinemia and insulin resistance in pregnant women (Chen et al., 2003). More striking, our laboratory found that overexpression of GPX1 in mice induced hyperglycemia, hyperinsulinemia, insulin resistance, and obesity at 6 months of age (McClung et al., 2004). To rescue these phenotypes and reveal the underlying mechanisms, we first employed diet restriction on the OE mice from 2 to 6 months of age (Wang

et al., 2008). Because this extended diet restriction eliminated all these type 2 diabetes-like phenotypes except for fasting hyperinsulinemia and hyper-secretion of insulin by glucose stimulation, these two abnormalities seemed to be the primary effect of GPX1 overproduction. To further eliminate these two phenotypes for a full rescue of these mice, we conducted a second experiment and employed dietary Se deficiency on the feed-restricted OE mice to preclude their GPX1 overproduction (Pepper et al., 2011). However, the dietary Se depletion showed no additional benefit, above that of the diet restriction alone, to the OE phenotypes. As metabolic impacts of the latter might be too drastic to allow demonstrations of the former effects on the OE phenotypes, such impacts of GPX1 activity exclusion should be tested in the full-fed OE mice.

Synthesis and secretion of insulin in pancreatic islets are regulated by transcriptional factors, signal molecules, and functional proteins encoded by genes such as *Pdx1*, *Beta2*, hnf1 homeobox a *(Hnf1a)*, *Foxa2*, v-maf musculoaponeurotic fibrosarcoma oncogene family protein a (*Mafa)*, *Glut2*, and *Gk1(Ahlgren et al., 1998; Gu et al., 2010; Lantz et al., 2004; Shih et al., 2001; Thorens et al., 1990; Winzell et al., 2011; Zhao et al., 2005)*. Hepatic lipogenesis is controlled by regulatory proteins and functional enzymes encoded by genes such as sterol regulatory element binding transcription factor 1a *(Srebp1a)*, sterol regulatory element binding factor 2 (*Srebp2)*, acetyl-coenzyme A carboxylase 1 (*Acc1)*, fatty acid synthase (*Fasn)*, and peroxisome proliferator-activated receptor gamma (*Pparγ)* (Jones et al., 2005; Mao et al., 2006; Paulauskis and Sul, 1988; Shimano, 2002). GK plays a key role in glucose oxidation (glycolysis), and phosphoenolpyruvate carboxykinase (PEPCK) is the rate-limiting enzyme in gluconeogenesis (Granner and Pilkis, 1990). As a major signal protein of cell death/survival, p53 is also involved in carbohydrate and lipid metabolism (Ashur-Fabian et al., 2010; Yahagi et al., 2004). We have

previously demonstrated distinct impacts of GPX1 knockout or overexpression on expression and function of many of these factors (Wang et al., 2011b; Wang et al., 2008). Plausibly, disorders of glucose and lipid metabolism in the OE mice might result from dys-regulations of those factors by the overproduction of GPX1. Therefore, our objective was to determine: 1) if a prolonged dietary Se depletion in the OE mice rescued their type 2 diabetes-like phenotypes; 2) if the presumed rescue was mediated by modulating expression and function of key factors related to insulin synthesis, secretion, and function in islets and lipogenesis, glycolysis, and gluconeogenesis in liver and muscle.

4.3 Materials and Methods

Mice, Diets, Physiological Tests, and Tissue Sample Collections

Our mouse experiments were approved by the Institutional Animal Care and Use Committee at Cornell University. Mice were reared in plastic cages in an animal room with constant temperature (22 ℃) and a 12 h light:dark cycle, and were given free access to feed and distilled water. The OE mice were derived from a B6C3 (C57B1×C3H) hybrid line (Taconic, Germantown, NY, USA) (Cheng et al., 1997). A total of 20 weanling male (1-month old) WT and OE mice (n = 5 for each genotype by diet) were fed a Torula yeast and sucrose based diet (TABLE B.) (Cheng et al., 1997) with Se-deficient (< 0.02 mg Se/kg, WT- and OE-) or Se-adequate (0.3 mg Se/kg as sodium selenite, WT+ and OE+) for 4 months, respectively. Mice were fasted 8 h overnight before all measures or experiments. Individual body weights, blood glucose concentrations, plasma insulin concentrations, insulin and glucose tolerances (ITT, 0.5 U insulin/kg body weight and GTT, 1g dextrose/kg), and glucose-stimulated insulin secretion (GSIS, 1 g dextrose/kg) of mice were recorded or determined at initial and then monthly. Blood

samples were collected via tail bleeding, blood glucose was determined by using a glucometer (Bayer, Elkhart, IN), and plasma insulin was determined by using a rat/mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL). At the end of study, all experimental mice were killed to collect blood (heart), pancreatic islets, liver, and muscle that were stored at -80^{0}C before analysis. The pancreatic islets were isolated and handpicked from mice using a standard procedure with minor modifications (Wang et al., 2008).

Molecular and Biochemical Assays

Total RNA was isolated from liver and pancreatic islets using a Trizol kit (Invitrogen, Carlsbad, CA) and the subsequent analysis by quantitative real-time PCR (Q-PCR) of 21 insulin synthesis and secretion-related genes in islets and 10 lipogenesis-related genes in liver were carried out as previously-described (German, 1993; Pepper et al., 2011). Each reaction was run in triplicate with validated primers listed in TABLE C. Liver lipid was extracted using a chloroform:methanol method and concentrations of TG, total cholesterol (TC), and non-esterified fatty acid (NEFA) were measured using commercial kits (Wako Chemicals, Richmond, VA). Hepatic GPX1 activity was measured at 25^{o}C using the coupled assay of NADPH oxidation as previously described (Lei et al., 1995). Hepatic GK activity was measured in a reaction coupled with glucose-6-phosphate dehydrogenase through monitoring production of NADPH (Danial et al., 2003). Liver and muscle PEPCK activities were determined using a spectrophotometric assay (Duff and Snell, 1982). Immunoblotting analysis of hepatic p53 protein was performed as described (Cheng et al., 1999a) using a primary rabbit antibody against mouse p53 protein (Cell Signaling, Danvers, MA), and relative density of the protein bands was

quantified using Canvas 7 software (Deneba Systems, Miami, FL). Protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL).

Statistical Analysis

Data were analyzed using SAS (release 6.11; SAS Institute, Cary, NC, USA). Dietary Se and genotype effects were tested by two-way ANOVA with (body weight and physiological data) or without (gene, protein, lipid, and enzyme data) time-repeated measurements. Data are presented as mean $\pm$ SE and significance was set at $P < 0.05$.

4.4 Results

4.4.1 Dietary Se Deficiency on Mouse Metabolic Phenotypes.

The OE mice had higher ($P < 0.05$) hepatic GPX1 activity than the WT mice, whereas dietary Se deficiency decreased ($P < 0.05$) the activity in both genotypes (Fig. 4.1A). The final body weight of OE mice was 27% heavier ($P < 0.05$) than the WT mice (Fig. 4.1B), and this genotype difference ($P < 0.05$) appeared as early as the first month (data not shown). However, dietary Se deficiency did not produce any or statistically-significant effect on mouse body weight at any time-point. In contrast, dietary Se deficiency decreased ($P < 0.05$) fasting blood glucose concentrations in both OE and WT mice and minimized their genotype difference at the final month (Fig. 4.1C). Dietary Se deficiency decreased ($P < 0.05$) post-mortem fasting plasma insulin concentration (Fig. 4.1D), and improved ($P < 0.05$) blood glucose tolerance or clearance in the OE mice (Fig. 4.2A). However, dietary Se deficiency impaired the early phase (15 and 30 min) of blood glucose clearance in the WT mice (Fig. 4.2A).

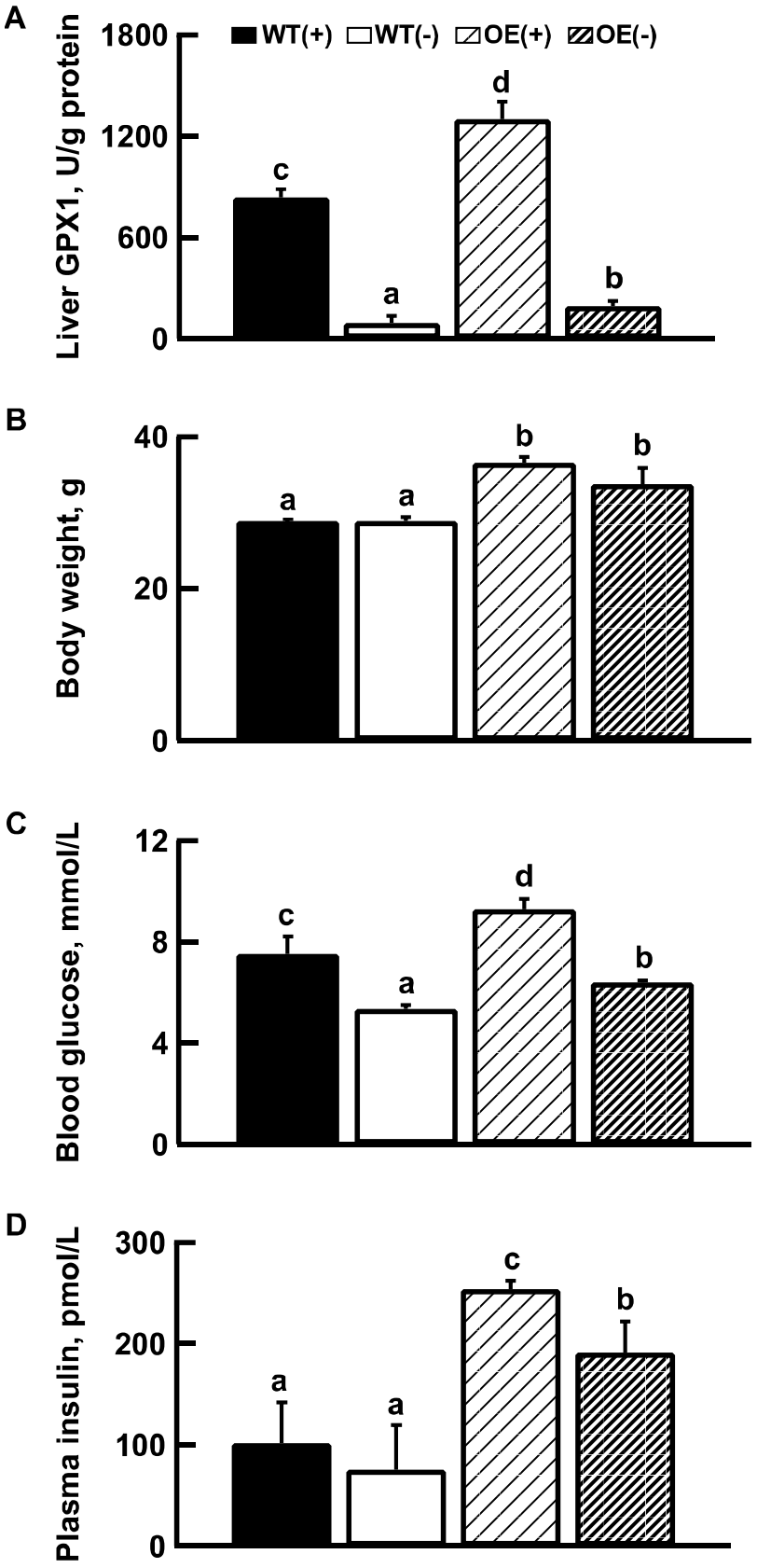

A
WT(+)
WT(-)
OE(+)
OE(-)
Liver GPX1, U/g protein
1800
1200
600
0
c
a
d
b
B
Body weight, g
40
20
0
a
a
b
b
C
Blood glucose, mmol/L
12
8
4
0
c
a
d
b
D
Plasma insulin, pmol/L
300
200
100
0
a
a
c
b

FIGURE 4.1 Effects of dietary Se concentration [deficiency as (-) and adequacy as (+)] on GPX1 activity, body weight, blood glucose and plasma insulin level.

(A) Hepatic GPX1 activity, (B) Body weight, (C) Fasting blood glucose concentration, and (D) Post-mortem fasting plasma insulin concentration in WT and OE mice at the end of experiment (5 months of age). Data presented as mean ± SE (n = 3 to 4 for panels A and D, n = 5 to 10 for panels B and C). Means for a given measure without sharing a common letter differ ($P < 0.05$).

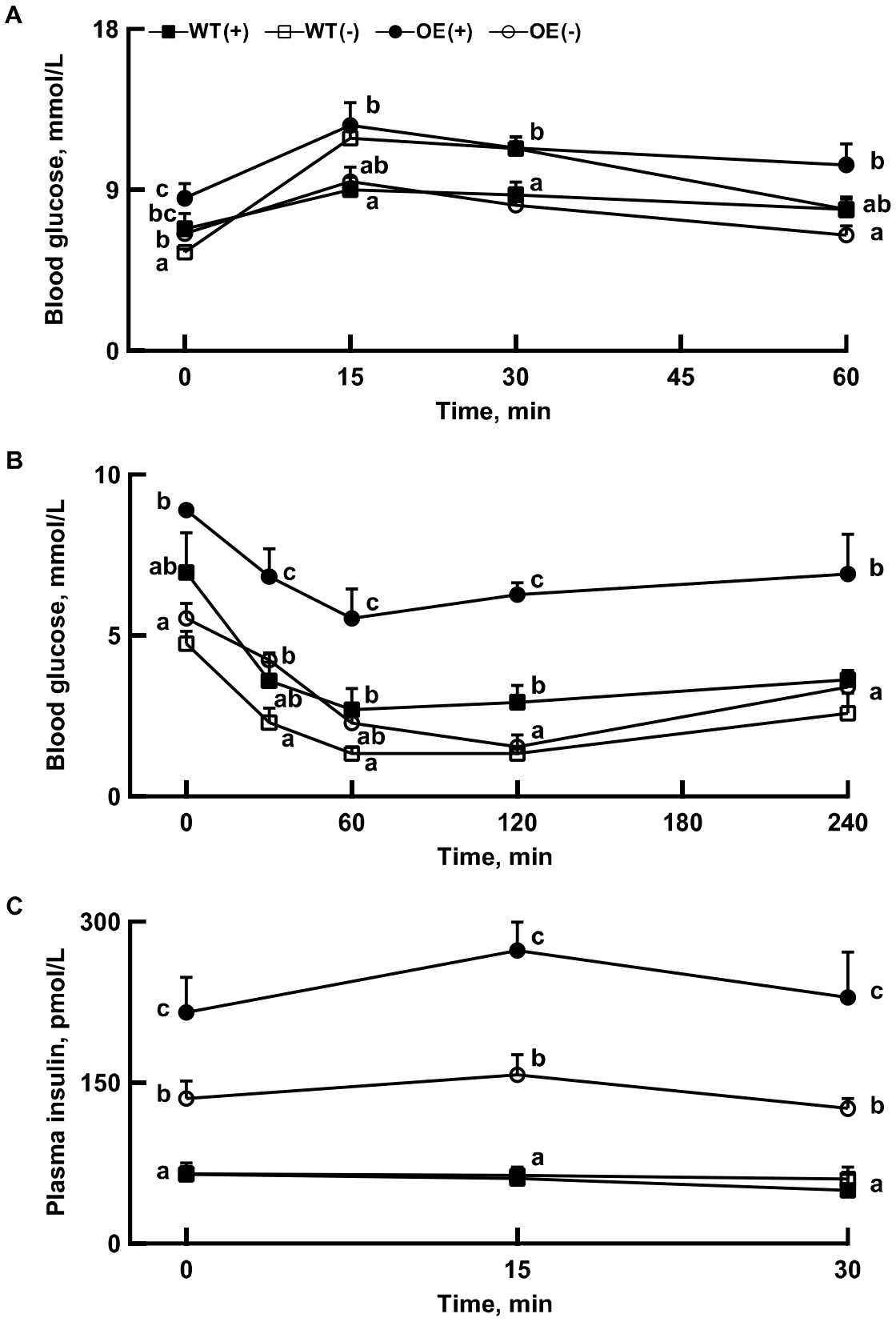

A
WT(+)
WT(-)
OE(+)
OE(-)
Blood glucose, mmol/L
18
9
0
0
15
30
45
60
Time, min
B
Blood glucose, mmol/L
10
5
0
0
60
120
180
240
Time, min
C
Plasma insulin, pmol/L
300
150
0
0
15
30
Time, min

FIGURE 4.2 Effects of dietary Se concentration [deficiency as (-) and adequacy as (+)] on glucose tolerance, insulin tolerance and insulin secretion.

(A) Body glucose tolerance (GTT, 1 g glucose/kg), (B) Body insulin tolerance (ITT, 0.5 U insulin/kg), (C) Glucose-stimulated insulin secretion (GSIS, 1g dextrose/kg) in WT and OE mice at 1 wk prior to the end of experiment. Data presented as mean ± SE (n = 5). Means for a given measure at a given time-point without sharing a common letter differ ($P < 0.05$).

Dietary Se deficiency improved ($P < 0.05$) insulin sensitivity at various time-points in both genotypes, and the magnitude of improvement was greater in the OE than the WT mice (Fig. 4.2B). Impacts of dietary Se and genotypes on the relative changes of body insulin and glucose tolerances were presented **in Supplemental Fig. 1**. Dietary Se deficiency attenuated ($P < 0.05$) plasma insulin concentrations at 0, 15, and 30 min after GSIS by 37, 42 and 45%, respectively, in the OE mice, but showed little effect in the WT mice (Fig. 4.2C). In Se adequacy, hepatic concentrations of TG, TC and NEFA (Fig. 4.3A, B, C) were 31, 105, and 37% greater ($P < 0.05$) in the OE than the WT mice, respectively. However, these genotype differences were either minimized or eliminated by dietary Se deficiency.

4.4.2 Dietary Se Deficiency on Islet Gene Expression.

Among the assayed genes in islets, 16 of them were affected ($P < 0.05$) by dietary Se and (or) genotype. Compared with their Se-adequate OE islets, the Se-deficient OE islets showed lower ($P < 0.05$) OE islet mRNA levels of *Beta2* and *Foxa2* and a decline trend ($P < 0.09$) of *Cfos* and *Pregluc*, but elevated ($P < 0.05$) mRNA levels of *Hnf1a, Hnf4α, Kir6.2 and Cat* (Fig. 4.4A). In the WT islets, dietary Se deficiency enhanced ($P < 0.05$) mRNA levels of *Cfos*, *Hnf4α*, *Foxo1*, *Gk1*, *Ins1*, and *p53* (Fig. 4.4A, B). Genotype differences between the Se-adequate WT and OE in islet mRNA levels of 6 genes (*Beta2*, *Cfos*, *Foxa2*, *Pregluc*, *Gk1* and *Sur1*) were removed by dietary Se deficiency (Fig. 4.4A, B). Neither genotype nor dietary Se showed significant effect on islet mRNA levels of *Irs2, Jund, Mafa, Sirt1,* or *Ucp2* (**Supplemental Fig. 2**).

4.4.3 Dietary Se Deficiency on hepatic Lipogenesis Gene Expression.

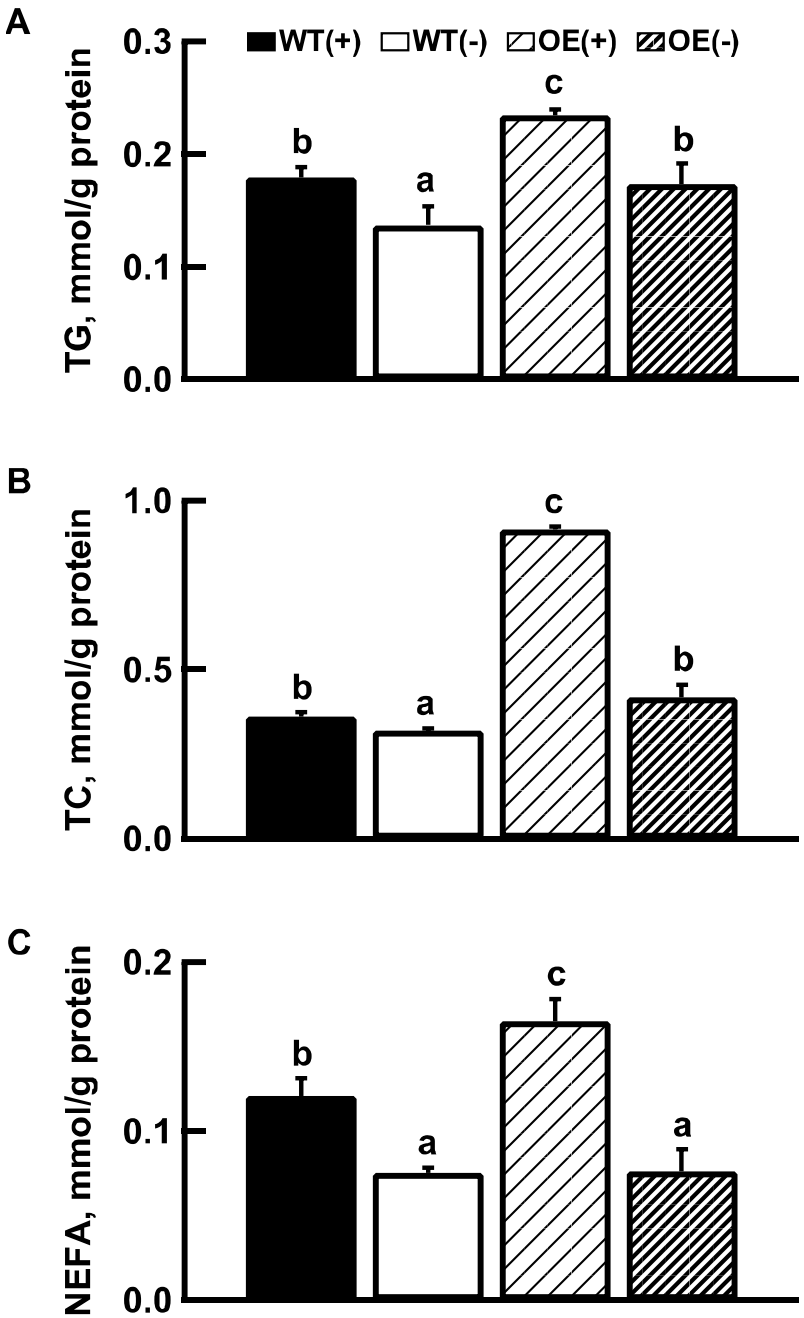


FIGURE 4.3 Effects of dietary Se concentration [deficiency as (-) and adequacy as (+)] on hepatic lipid profiles. Hepatic concentrations of (A) TG, (B) TC, and (C) NEFA in WT and OE mice at the end of experiment (5 months of age). Data presented as mean ± SE (n = 5). Means for a given measure without sharing a common letter differ ($P < 0.05$).

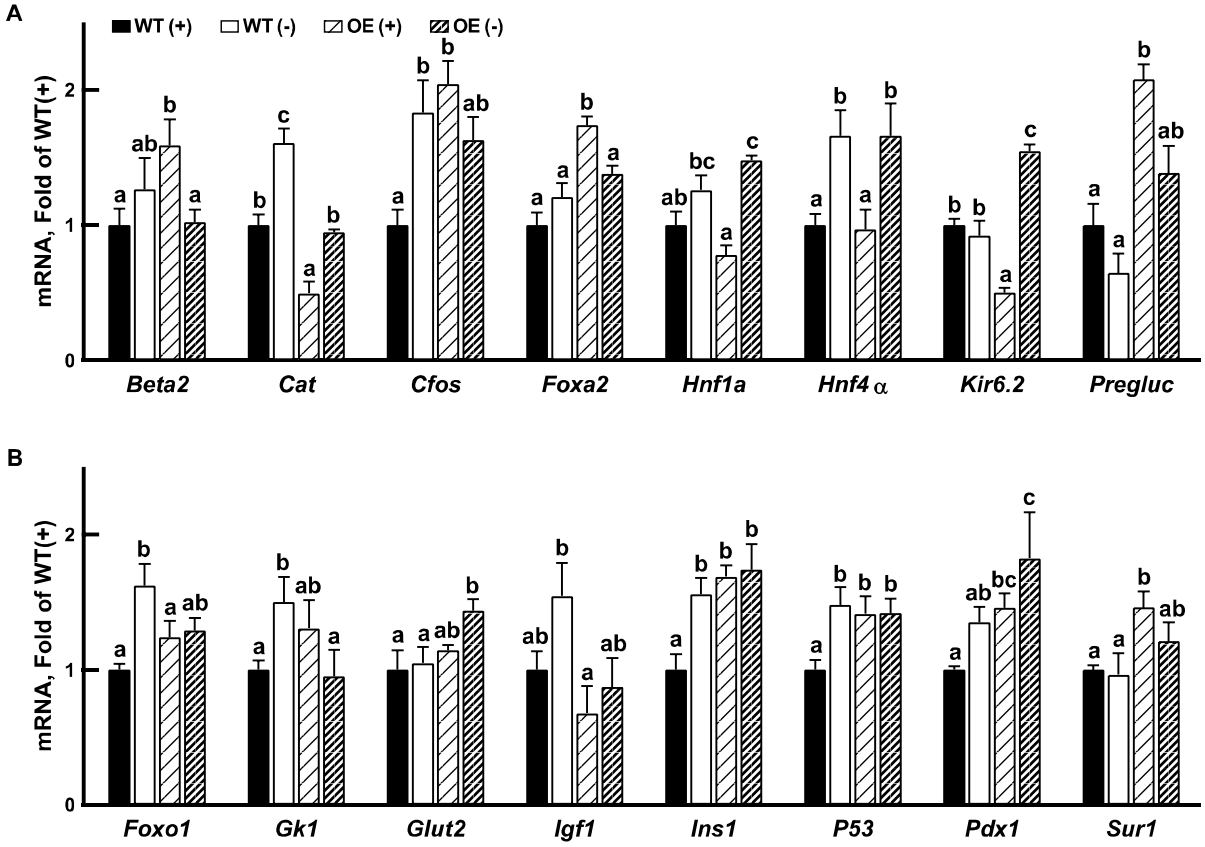


FIGURE 4.4 Effects of dietary Se concentration [deficiency as (-) and adequacy as (+)] on pancreatic islet mRNA abundances of insulin-related genes in WT and OE mice at the end of experiment (5 months of age). Panel A shows genes in the OE islets that were responsive to dietary Se deficiency, whereas panel B shows those less responsive to the same treatment. Data presented as mean ± SE (n = 5). Means for a given gene without sharing a common letter differ ($P < 0.05$).

Hepatic *Gk1* mRNA abundance was 62-fold greater ($P < 0.01$) in the Se-adequate OE than the Se-adequate WT mice, while dietary Se deficiency produced ($P < 0.05$) opposite changes of the *Gk1* expression between the two genotypes (Fig. 4.5). Meanwhile, dietary Se deficiency increased ($P < 0.05$) *Pparγ* in the OE mice, but decreased ($P < 0.05$) *Srebp1a* and *Srebp2* in the WT mice and *Acc1* in the OE mice (Fig. 4.5). In addition, the OE mice displayed greater ($P < 0.05$) mRNA levels of *Acc1*, *Cyp7a1*, *Fasn*, *Mccc1*, and *Pparγ*, but lower ($P < 0.05$) mRNA levels of *F1,6bp* and *Hmgcs2* than the WT mice (Fig. 4.5).

4.4.4 Dietary Se Deficiency on Tissue GK and PEPCK Activities and p53 Protein.

Hepatic GK activity in the Se-adequate OE mice was 2.5-fold greater ($P < 0.05$) than that of the Se-adequate WT mice (Fig. 4.6A). Dietary Se deficiency suppressed ($P < 0.05$) the enzyme activity in the OE mice to the level similar to that of the WT mice. Likewise, muscle PEPCK activity was 6-fold ($P < 0.05$) greater in the Se-adequate OE mice than in the Se-adequate WT mice (Fig. 4.6B), and dietary Se deficiency decreased ($P < 0.05$) the enzyme activity by 38% in the OE mice. Hepatic PEPCK activity was decreased ($P < 0.05$) by dietary Se deficiency in the WT. Liver p53 protein in the Se-deficient WT was 1.2-fold higher ($P < 0.05$) than that in the Se-adequate WT (Fig. 4.6C).

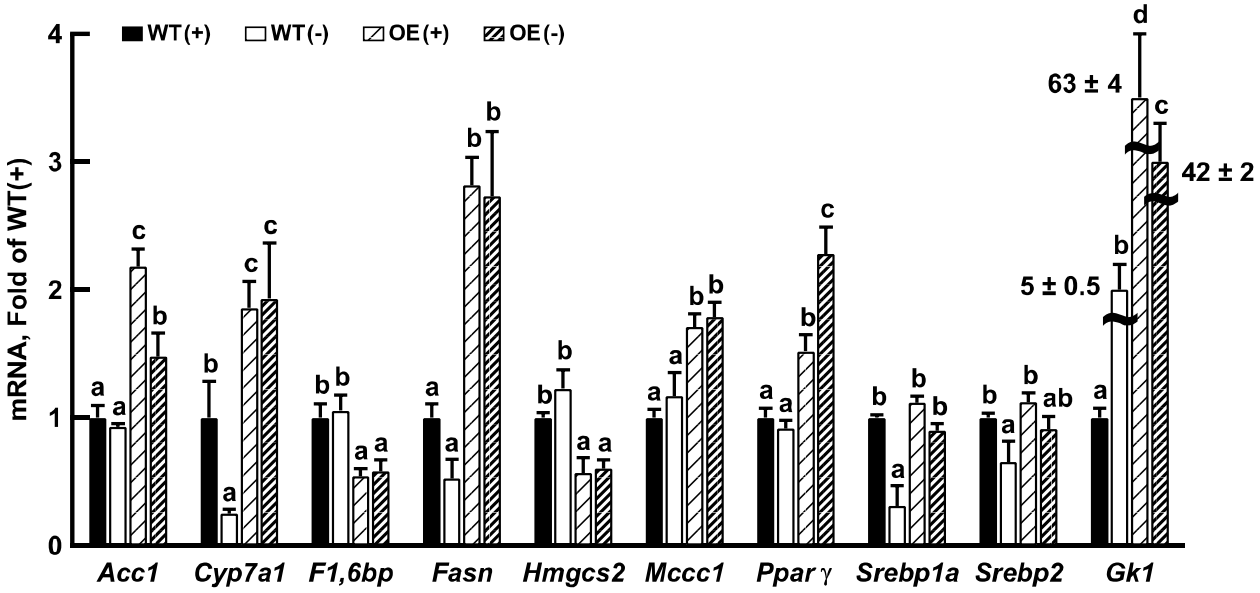


FIGURE 4.5 Effects of dietary Se concentration [deficiency as (-) and adequacy as (+)] on hepatic mRNA abundances of lipogenesis-related genes in WT and OE mice at the end of experiment (5 months of age). Data presented as mean ± SE (n = 5). Means for a given gene without sharing a common letter differ ($P < 0.05$).

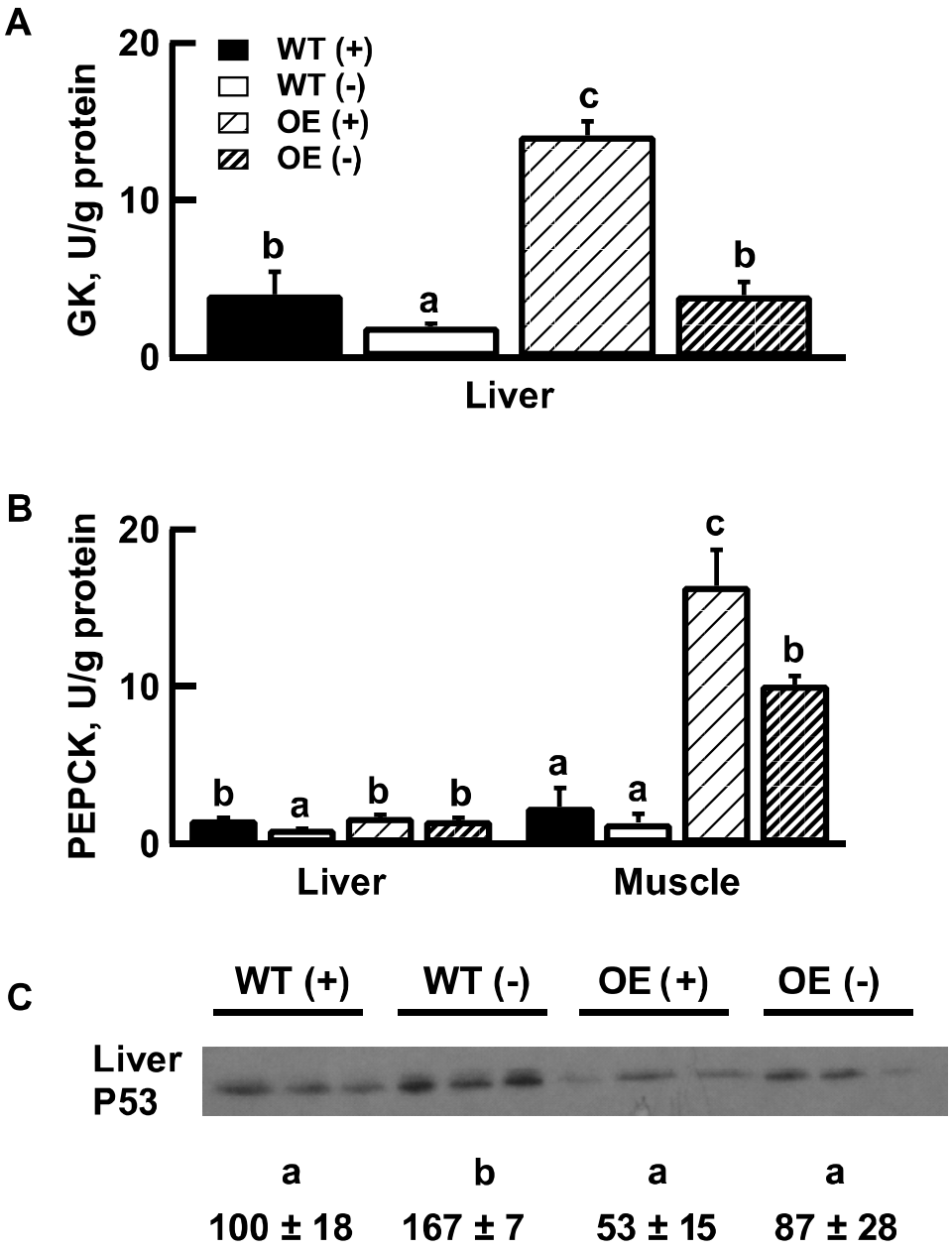


FIGURE 4.6 Effects of dietary Se concentration [deficiency as (-) and adequacy as (+)] on glucose homeostasis-related enzyme activities and P53 protein level. (A) Hepatic GK activity, (B) Hepatic and muscular PEPCK activities, and (C) Hepatic P53 protein in WT and OE mice at the end of experiment (5 months of age). Data presented as mean ± SE (n = 4 to 5) and the image (C) was a representative of 4 independent experiment. Means for a given measure without sharing a common letter differ ($P < 0.05$).

4.5 Discussion

The present study represents the third experiment out of a series of attempts to rescue the type 2 diabetes-like phenotypes in the OE mice. After we obtained a partial rescue of these phenotypes by diet restriction (Wang et al., 2008) and no additional benefit of dietary Se deficiency in the diet-restricted OE mice (Pepper et al., 2011), this study was conducted to explore if dietary Se deficiency in the full-fed OE mice could completely rescue their phenotypes. While dietary Se deficiency indeed precluded the GPX1 overproduction in the OE mice and thus minimized their GPX1 activity difference from the WT mice, three of their phenotypes including hyperglycemia, insulin resistance, and elevated hepatic lipid profiles (McClung et al., 2004) were nearly rescued. Meanwhile, their hyperinsulinemia and aggravated GSIS were also improved by dietary Se deficiency. Interestingly, the overall impact of dietary Se deficiency in the full-fed OE mice resembled that by diet restriction in Se-adequate OE mice (Wang et al., 2008). Because these two treatments produced distinct primary effects, it is fascinating for us to see indistinct phenotype responses to them. Specifically, diet restriction in the Se-adequate OE mice eliminated obesity and brought their body weight down to a level lower than the WT mice, but had little effect on activities of GPX1 and other two selenoproteins in liver, pancreas, muscle, and plasma (Wang et al., 2008). In contrast, dietary Se deficiency in the full-fed OE mice in the present study decreased their liver GPX1 activity, but did not alter their body weight. Thus, diet restriction alleviated the OE phenotypes by mechanisms related to the obesity, whereas the actions of dietary Se deficiency were mediated by precluding the overproduction of GPX1 activity and perhaps down-regulation of other selenoproteins as well (Pepper et al., 2011; Wang et al., 2008). Intriguingly, these two treatments showed no additive benefit on the OE phenotype in our second experiment of the series (Pepper et al., 2011). In that case, diet restriction seemed to be too

drastic to allow a demonstration of metabolic impact of dietary Se deficiency. Furthermore, neither dietary Se deficiency from 1 to 5 months of age nor diet restriction from 2 to 6 months of age could completely rescue hyperinsulinemia or aggravated GSIS. Likely, these two disorders were pre-disposed by GPX1 overproduction in prenatal or neonatal stages.

Two "pro-insulin" genes: *Beta2* (Gu et al., 2010; Malecki et al., 1999) and *Foxa2* (Lantz et al., 2004), were decreased by dietary Se deficiency in the OE mice. Because these two genes encode key transcriptional factors for beta cell differentiation and insulin synthesis (Gu et al., 2010; Lee et al., 2002), down-regulations of their mRNA and presumably their functional protein levels in the OE mice were consistent with their hypoinsulinemic response to dietary Se depletion. Meanwhile, dietary Se deficiency also removed the genotype differences between the Se-adequate WT and OE islets in another 4 genes (*Cfos, Pregluc*, *Gk1*, and *Sur1*) related to insulin synthesis and secretion. These changes offer potential mechanistic explanations to the attenuated hyperinsulinemia and improved GSIS in the Se-deficient OE mice. Physiological implications remain unclear to us for the elevated islet *Hnf1a, Hnf4α,* and *Kir6.2* mRNA levels by dietary Se deficiency in the OE mice. Up-regulation of these pro-insulin synthesis and secretion genes may be perceived as a feedback response to the improved metabolic condition and(or) down-regulation of other pro-insulin genes. Among the 6 genes up-regulated by dietary Se deficiency in islets of the full-fed WT mice, 2 of them (*Ins1*and *Gk1*) were also enhanced by dietary Se deficiency in islets of the restricted-fed WT mice (Pepper et al., 2011). Thus, *Ins1* and *Gk1* are highly responsive to dietary Se supply or body Se status.

The improved hepatic lipid profile in the Se-deficient OE mice, compared with their Se-adequate controls, was concurrent with a down-regulation of the GK mRNA level and activity. Being an insulin-dependent key factor involved in lipogenesis (Foretz et al., 1999), GK activity is closely related to energy production through glycolysis and reactions in tricarboxylic acid cycle (Matschinsky, 1996), and consequently provides substrate precursors such as acetyl-CoA and NADPH for biosynthesis of cholesterol and other lipids. In Se adequacy, hepatic GK mRNA level and activity were over 62- and 2.5-fold greater in the OE than the WT mice, respectively. Clearly, the suppression of hepatic GK activity by dietary Se deficiency in the OE mice to the level of WT mice helped down-regulate that pathway of lipogenesis. Meanwhile, down-regulation of hepatic *Acc1* in the Se-deficient OE mice was also attributed to their decreased hepatic lipid profiles because ACC1 is a rate-limiting enzyme of lipogenesis that is activated by insulin (Scott et al., 2012). The elevated hepatic *Pparγ* expression in the Se-deficient OE mice was hard to link to their hepatic lipid status, but the decreased muscle PEPCK activity was consistent with the hypoglycemic response to dietary Se deficiency, presumably by decreasing gluconeogenesis (Yang et al., 2009). Meanwhile, the hypoglycemic and lowering hepatic lipid profile effect of dietary Se deficiency in the WT mice was associated with a down-regulation of GK and PEPCK activities and an up-regulation of p53 protein in liver. While key roles of GK in glycolysis and PEPCK in gluconeogenesis are well-documented (Granner and Pilkis, 1990), the tumor suppressor p53 protein has been revealed recently as a novel regulator of lipid metabolism pathways that is involved in the development of obesity and fatty liver diseases (Ashur-Fabian et al., 2010; Yahagi et al., 2004). Long-term dietary selenomethionine consumption has been shown to increase exon-specific DNA methylation of *p53* gene in a Se-dose-dependent manner in rat liver (Zeng et al., 2011). Thus, the elevated hepatic p53 protein in the Se-deficient WT

mice not only helps explain the attenuated lipogenesis, but also unveils a novel link between Se/GPX1 and p53 on glucose and lipid metabolism. In addition, an increased hepatic *Gk1* mRNA in the Se-deficient WT mice might be perceived as a feedback mechanism in response to the declined GK activity (Jackson et al., 2000). Meanwhile, down-regulation of hepatic *Srebp1a* and *Srebp2* mRNA in the Se-deficient WT mice was also supposed to inhibit lipogenesis because these two genes encode key transcriptional factors of lipogenesis (Edwards et al., 2000).

In summary, dietary Se deficiency partially disallowed the GPX1 overproduction in the full-fed OE mice and nearly rescued all of their type 2 diabetes-like phenotypes. As proposed in Fig. 4.7, the observed improvement was associated with down-regulations of "pro-insulin synthesis and secretion" genes in islets, lipogenesis rate-limiting enzyme genes in liver, and key glycolysis and gluconeogenesis enzymes in liver and(or) muscle. Although molecular or physical implications for certain changes of these assayed genes remain elusive, our study have demonstrated a strong regulation of the hepatic GK mRNA and activity by GPX1 overproduction and dietary Se deficiency, and a novel link of Se/GPX1 to p53 on body energy metabolism. Despite the focus on GPX1 role in the OE phenotype in the present study, other Se-dependent proteins including selenoprotein P (Misu et al., 2010; Speckmann et al., 2008), methionine-R-sulfoxide reductase B1 (Labunskyy et al., 2011), thioredoxin reductase 3 (Labunskyy et al., 2011), and selenoprotein S (Gao et al., 2004) have also been shown to be involved in glucose metabolism or diabetes. Because expressions of these proteins are affected by dietary Se (Labunskyy et al., 2011; Zeng et al., 2012), and to a certain extent by the GPX1 overproduction (Labunskyy et al., 2011), our ongoing research is assessing their relative roles in the dietary Se-mediated changes of the OE phenotypes.

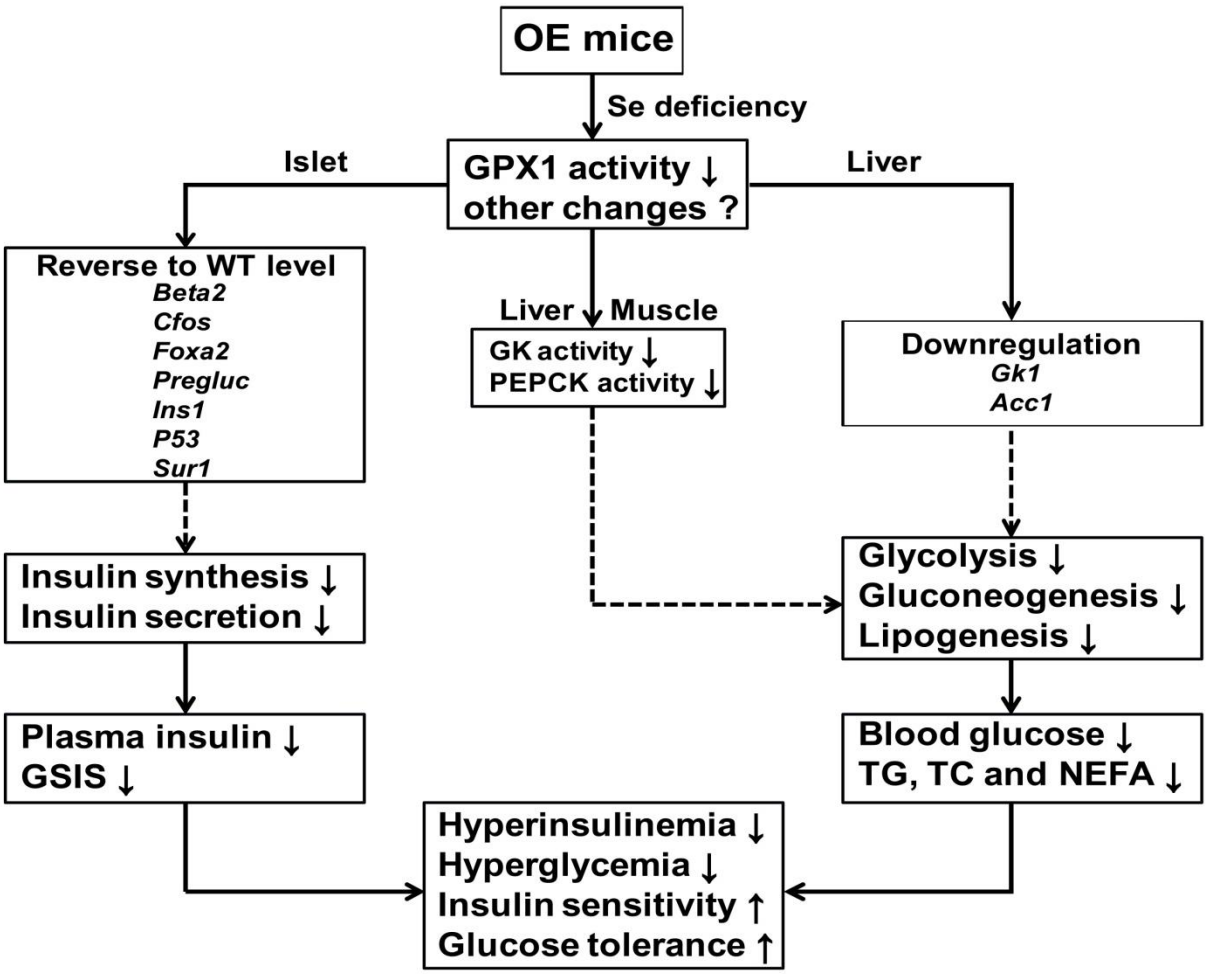


FIGURE 4.7 Scheme of the regulation of dietary Se deficiency on the T2DM-like phenotypes in OE mice.

CHAPTER 5

General Discussion and Future Directions

5.1 General Discussion

OE mice have developed hyperinsulinemia, hyperglycemia, insulin resistance, and obesity (McClung et al., 2004), and their islets have exhibited diminished *Reg2* gene expression (Fig. 1.5A, B). The overall research objective for this dissertation is to understand how GPX1 overexpression led to the T2DM-like phenotypes in mice, with the long-term goal of innovative drug development to treat T2DM in humans and to improve human life.

In Chapter 2, studies were conducted to investigate whether or not the ROS and GSH are the mediator of downregulation of the REG2 protein by GPX1, and whether or not exogenous REG2 administration in OE mice has any therapeutic effect on the OE T2DM-like phenotypes. To address these questions, we firstly examined REG2 protein change in the islet and pancreas after changing the ROS level *in vitr*o and *in vivo*. We observed a down-regulated REG2 protein level in islets treated with antioxidants: ebselen, NAC or Na_2SeO_3, and observed up-regulated REG2 protein expression in islets or pancreas that had increased oxidative stress after H_2O_2, STZ or DQ treatment. Next, we expressed and purified the recombinant mouse REG2 protein from yeast and i.p. injected it into OE mice to examine the potential therapeutic role of REG2. Strikingly, fifteen-minute pre-injection of REG2 protein decreased glucose tolerance and glucose-stimulate insulin secretion in OE mice. Two-week daily exogenous REG2 protein administration decreased fasting blood glucose, plasma insulin, GSIS, glucose tolerance, body weight, and plasma TG, but not insulin tolerance. Taken together, we demonstrated that GPX1

downregulated REG2 by modulating the redox state. The depletion of islet Reg2 by GPX1 overexpression mediated some of the OE T2DM-like phenotypes, as injection of REG2 in OE mice alleviated hyperinsulinemia, hyperglycemia and hypertriglycemia.

In Chapter 3, the research was mainly focused on studying the molecular mechanisms underlying how REG2 decreased insulin secretion *in vitro*. Firstly, we confirmed that REG2 inhibited insulin secretion in a glucose-dependent way, as GSIS was decreased by the REG2 pre-treatment, but not the KCL-stimulated or arginine-stimulated insulin secretion. These results suggested that glucose metabolism-related pathways might be the main target for REG2 in the OE islets. To test this hypothesis, we examined the change of the key regulators in the pathway after REG2 treatment. As expected, we observed a decrease in glucose metabolism-related genes such as *Glut2, Gk1, Pklr, Atp5b, Cav1.2, Cav1.3* and *Prkcb*, but not in insulin synthesis-related genes, such as *Ins1, Foxa2, Beta2 and Pdx1*. We also detected decreases in GLUT2 protein level, GK activity, and mitochondria membrane potential, as well as an increase in UCP2 protein level after REG2 treatment in OE islets. Secondly, we found that the ligand-binding residues in the CTLD might be the functional domain that enabled the REG2 protein to decrease GSIS in islets, and not the acidic residues that were predicted to bind to $CaCO_3$. This conclusion was supported by the fact that REG2 with mutation in CTLD failed to suppress GSIS, while the REG2 with mutation in the acidic residues showed similar GSIS inhibition effects as the native REG2 protein. These findings strongly suggested that the depleted Reg2 in OE islets played an important role in development of T2DM-like phenotypes in OE mice. This study opens new doors to bridge two "unrelated" fields and expands the function of GPX1 as an antioxidant. In

addition, it provides new insights into a therapeutic strategy for T2DM, in particular the prediabetic state in human.

In Chapter 4, the objective was to determine whether or not dietary Se deficiency could preclude GPX1 overproduction and rescue the T2DM-like phenotypes in full-fed OE mice. After a 4-month feeding trial, OE mice fed a Se deficiency diet showed significant reduction of liver GPX1 activity, improved hyperinsulinemia, hyperglycemia, insulin sensitivity, and glucose tolerance when compared to the OE mice fed a Se adequate diet. These alleviated T2DM-like phenotypes could be explained by the decreased expression of "pro-insulin synthesis and secretion" genes in islets as well as decreased insulin synthesis and secretion level; downregulation of protein activity of the key enzymes that regulate glycolysis and gluconeogenesis and decreased blood glucose, such as GK and PEPCK.

In summary, the major discoveries of this dissertation are as follows:

1. New insights into the biochemical regulation of REG2 by GPX1.
2. First-time identification of the downstream signaling pathway of REG2 and the possible functional domain of REG2 protein.
3. Novel therapeutic potential of REG2 and dietary Se manipulation in treating T2DM, which may lead to innovative drug discovery.

5.2 Future Directions

Future studies will focus on identifying the REG2 putative receptor in islets and how it interacts and inhibits the glucose metabolism pathway to regulate GSIS. Although we have identified

some interacting protein candidates using immunoprecipitation and mass spectrometry, we have not been able to confirm the one-to-one direct binding by using either AP-TAG binding assay or yeast two hybrid approach. Different binding conditions may be used to test the binding of REG2 and its interacting partners, such as changing the concentration of glucose or $CaCl_2$ in the AP conditioned medium. Meanwhile, alternative approaches can be used, such as Co-immunoprecipitation or Bimolecular fluorescence complementation.

Although we did not identify calcium channel, voltage-dependent, L type, alpha 1C subunit (Cav1.2) from our IP-MS study, Cav1.2 can be another REG2 binding candidate based on its physiological function in insulin secretion and its binding capability to lectins. L-type voltage-gated calcium channel plays important roles in GSIS by activating Ca^{2+} influx upon membrane depolarization. One of the isoforms, Cav1.2 determines the main electrophysiological and pharmacological properties of the channel. Deletion of Cav1.2 in mice significantly reduces first phase insulin release (Schulla et al., 2003), while knockdown Cav1.2 in INS-1 832/13 cells impaired GSIS (Nitert et al., 2008). Regulation of Cav1.2 cluster trafficking to cell surface is one of the mechanisms that Cav1.2 regulate β-cell Ca^{2+} homeostasis and GSIS (Buda et al., 2013). Cav1.2 was also found to interact with lectins (Wang et al., 2011a). Galectin-1 (Gal-1) is beta-galactoside-binding lectin and a carbohydrate-binding protein that can inhibit Cav1.2 current via interacting with Cav1.2 and reducing the functional surface expression of Cav1.2 (Wang et al., 2011a). The α1c I-II loop of Cav1.2 is involved in the binding of Gal-1. Since REG2 has a conserved C-type lectin domain, this domain may enable REG2 to interact with Cav1.2. To test the hypothesis, we can use the AP-TAG binding assay or Co-immunoprecipitation method to determine the physical interaction between REG2 and Cav1.2.

APPENDIX 1

TABLE B. Composition of Torula yeast basal diet

Ingredient	Percentage of diet
Torula yeast[2]	40.00
Sucrose[3]	52.20
Stripped corn oil[3]	5.00
DL-Methionine[3]	0.50
Vitamin mix[4]	0.20
Mineral mix[5]	2.10

1. Diet is supplemented Se (Na_2SeO_3) at < 0.02 mg/kg or 0.3 mg/kg.

2. Torula yeast was purchased from Rhinelander Paper Compnay, Inc., Lake States division (Rhinelander, WI), and contained less than 0.02 mg Se/kg by analysis.

3. Sucrose, corn oil, and DL-methionine were purchased from Dyets Inc. (Bethlehem, PA).

4. Vitamin mix components (per kg diet): all-*trans* retinyl palmitate, 10.0 mg; cholecalciferol, 45.0 μg; menadione sodium bisulfite, 1.2 mg; biotin, 0.2 mg; vitamin B-12, 0.015 mg; d-calcium-pantothenate, 30.0 mg; folacin, 4.0 mg; niacin, 50.0 mg; pyridoxine HCl, 10.0 mg; riboflavin, 10.0 mg; and thiamine HCl, 10.0 mg.

5. Mineral mix components (per kg diet): CaCO3, 19.69g; NaCl, 1.2g; MnSO4, 60 mg; KCO3, 1.0 mg; MgSO4*7H2O, 6.15g; FeSO4*7H2O, 0.5h; ZnO, 0.1g; CuSO4*7H2O, 20.0mg; CoCl2*6H2O, 3.34mg; NaMoO4*2H2O, 6.7mg; and Ni(NO3)2*6H2O, 15.0mg.

APPENDIX 2

TABLE C. Q-PCR Primers

Gene Name	For (5'---3')	Rev (5'---3')
ISLETS		
Atp5b	CTGATAAGCTGGCAGAAGAGCA	CTGAGCTCTCGCTTGATATGGA
Beta2	GCCCAGCTTAATGCCATCTTT	CAAAAGGGCTGCCTTCTGTAA
Cat	TCCAGTGCGCTGTAGATGTGAA	TGTTCTCACACAGGCGTTTCCT
Cav1.2	CACTGGAGCAAGGGTTCAATA	GATGCTCAACCAAACTCGAAAG
Cav1.3	GAAGCACCTTTGACAACTTTCC	ATCACCGCATTCCAGTCTTC
Cfos	ATTTGACTGGAGGTCTGCCTG	CACGTTGCTGATGCTCTTGAC
Foxa2	TGCCCCCTACGCCAACATGA	TGGCGTGTGTGTAGCTGCGT
Foxo1	GCTGCAATGGCTATGGTAGGA	GTCACAGTCCAAGCGCTCAAT
Gk1	CTGTTAGCAGGATGGCAGCTT	TTTCCTGGAGAGATGCTGTGG
Glut2	CTCCAGGAAGGGTGCTAAACC	TGCTCCCTATCCGTTCTTCAA
Hnf1a	GTGTAACTGCACAGGAGGCAAA	TTCTCACGTGTCCCAAGACCTA
Hnf4	GCCACAGTTTTCCACCAAGAG	AAGGAGGACGTCTGCTTCTGA
Hprt	GGCCAGACTTTGTTGGATTTG	TGCGCTCATCTTAGGCTTTGT
Igf1	CAGGGATGAAGAAGGAGCAGA	TAAGTGCCGTATCCCAGAGGA
Ins1	AGGACCCACAAGTGGAACAAC	GTGCAGCACTGATCCACAATG
Irs2	GCCTGGGGATAATGGTGACTA	TCCATGAGACTTAGCCGCTTC
Jund	CGAGCAGCAGCATGCTGAAGA	GCCGACCCTGGTTTCAA
Kir6.2	CTGTCCCGAAAGGGCATTAT	CGTTGCAGTTGCCTTTCTTG
Mafa	GAGGAGGTCATCCGACTGAAA	GCACTTCTCGCTCTCCAGAAT
p53	GACCCTGGCACCTACAATGAA	GGGTGGATAAATGCAGACAGG
Pdx1	CTTAACCTAGGCGTCGCACAA	GAAGCTCAGGGCTGTTTTTCC

Pklr	ATCTGGTGATTGTGGTGACAGG	GGGGTGTGGGTTGAAAGAAA
Pregluc	TGAGATGAGCACCATTCTGGA	TCCGCAGAGATGTTGTGAAGA
Prkcb	CAAGGAGATTCAGCCACCTTAT	TTTGTCAGTGGGAGTCAGTTC
Reg1	GACTCCATGATCCCAAAAGG	CACAGTAGCCACGATTGGAA
Reg2	TCCCCTTGGCTGAAAAAGAC	CCAGGTCAAACGGTCTTCAA
Sirt1	TGGATTTGGAGCTGATGGAG	CAATGCTGGTGGAGCAATTC
Sur1	TCAACTTGTCTGGTGGTCAGC	GAGCTGAGAAAGGGTCATCCA
Ucp2	GGGTTCATGCCTTCCTTTCT	AGATTGGTAGGCAGCCATTAGG
LIVER		
Acc1	TGAGGAGGACCGCATTTATC	GCATGGGATGGCAGTAAGGT
Cyp7a1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA
F1,6bp	CTTGCCATGGATTGTGGTGT	TTGATGGCAGGGTCAAAGTC
Fasn	AAGGCTGGGCTCTATGGATT	TGAGGCTGGGTTGATACCTC
Gk1	CTGTTAGCAGGATGGCAGCTT	TTTCCTGGAGAGATGCTGTGG
Hmgcs2	CCGTATGGGCTTCTGTTCAG	AGCTTTGTGCGTTCCATCAG
Mccc1	GGAAAATCGGGTATCCTGTTATG	AGACTTCTTCGCCTCCCTCC
Pparγ	CCGTGATGGAAGACCACTCG	CAGGTCATATTTGTAATCAGCAACC
Srebp1a	GGAACAGACACTGGCCGAGA	GCATAGGGGGCGTCA
Srebp2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA

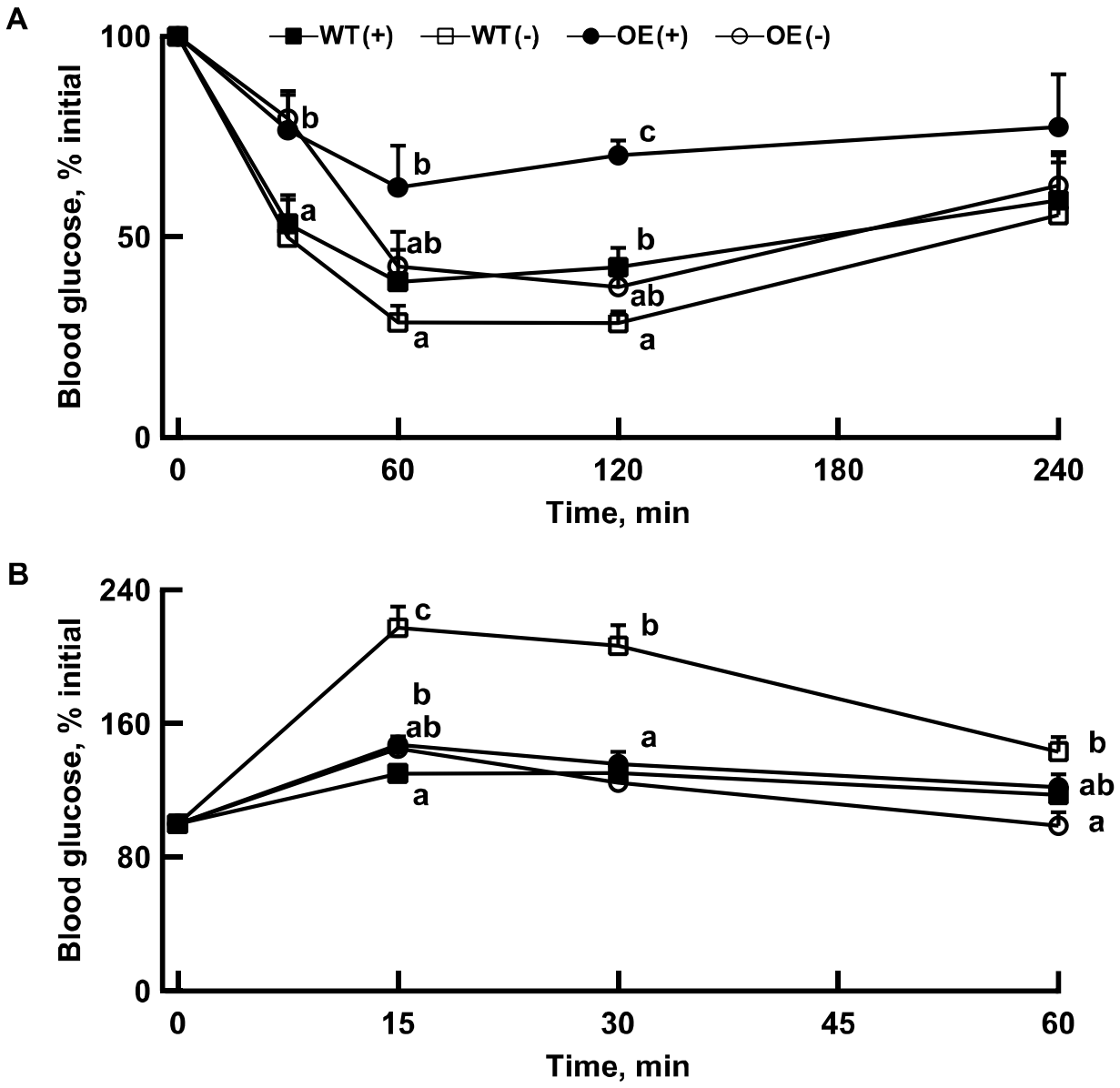
APPENDIX 3

TABLE D. *Reg2* mutagenesis primers

M1: G142A	ctgccaaccgtgCttattgtgtatcgctgacttc
M1: G142A	atacacaataaGcacggttggcagtgcttggagc
M1: K157A, D158A	aaaaatggGCTgCcgaaaactgtgaggcacag
M1: K157A, D158A	cagttttcgGcAGCccatttttgtatgctgtgtttgtttgaag
M2: E59G, D60G, E66G	atctaattgGagGAcgtttgacctggggTgGTgctgatctcttttgccagaac
M2: E59G, D60G, E66G	gagatcagcACcAccccaggtcaaacgTCctCcaattagataataacagtaggaacc
M2: D158G, E159G, E162G	aatggaaggGTgGaaactgtgGggcacagtactcctttgtc
M2: D158G, E159G, E162G	tactgtgccCcacagtttCcACccttccatttttgtatgctgtg

APPENDIX 4

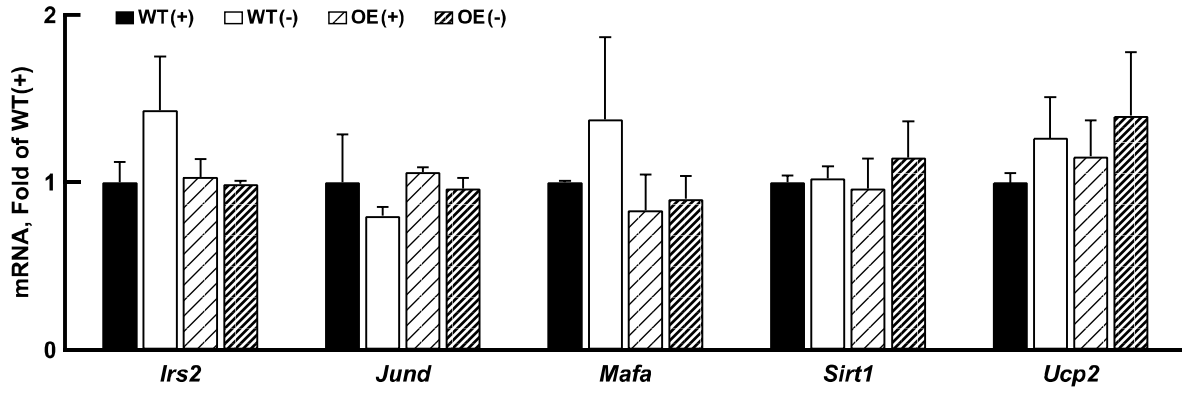
Supplemental FIGURE 1



Effects of dietary Se concentration [deficiency as (-) and adequacy as (+)] on (A) body insulin tolerance (ITT, 0.5 U/kg) and (B) glucose tolerance (GTT, 1 g/kg). Data were expressed as relative percentages of the respective initial (0 min) values, and are presented as means ± SE (n = 4-5). Values at a given time-point not sharing a common letter differ ($P < 0.05$).

APPENDIX 5

Supplemental FIGURE 2



Effects of dietary Se concentration [deficiency as (-) and adequacy as (+)] on pancreatic islet mRNA abundances of insulin-related genes in WT and OE mice at the end of experiment (5 months of age). Data were presented as mean ± SE (n = 5).

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